Recd from M/S Kothari

The Sections and the Division hold meetings regularly, one evening each month, during the academic year, October to May, inclusive. All meetings are held at the building of The New York Academy of Sciences, 2 East Sixty-third Street, New York 21, New York. Two-day conferences are also held at irregular intervals.

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Editor
ROY WALDO MINER

BIOLOGY OF POLIOMYELITIS*

Conference Chairman and Consulting Editor
KARL HABEL

CONTENTS

*This series of papers is the result of a conference on *Biology of Poliomyelitis* held by the Section of Biology of The New York Academy of Sciences January 20 and 21, 1955

INTRODUCTION

By Hilary Koprowski*
Lederle Laboratories Division, American Cyanamid Company, Pearl River, N Y.

Although poliomyelitis has already received a great deal of attention, it is of a complexity and importance that demand continued study from a great variety of approaches, and the reports made in this monograph represent a wide range of investigation. The results obtained by one worker often illuminate and extend the comprehension of another, and that, it is hoped, will be one of the accomplishments of this publication

At the outset, it may be well to consider the views of Aristotle, who, as far as we know, was the first to proclaim explicitly that man is a rational animal His reason was one which does not seem very impressive today. It was that some people can do sums Bertrand Russell, in his essay *An Outline of Intellectual Rubbish*,[1] commenting upon Aristotle's axiom, said "Throughout a long life, I have looked diligently for evidence in favor of this statement (that man is a rational animal) but so far I have not had the good fortune to come across it, though I have searched in many countries spread over three continents " It is not certain whether Bertrand Russell is familiar with poliomyelitis research It is certain Aristotle was not If the latter were alive today, would he find in the fact that men do research in the field of poliomyelitis a good reason to place them in the category of rational animals? It is not easy to reply to that question in the affirmative without some reservation.

The feeling of mistrust expressed occasionally by scientists working on poliomyelitis towards concepts of research other than their own leads one to wonder if the remarks of the great French poet Paul Valery about history might not be applied to this field Valery described history as "the most dangerous product that the chemistry of the intellect has invented Its properties are well known It engenders dreams, it intoxicates the workers, it begets false memories, keeps their old wounds open, disturbs their sleep, leads them to [illegible]

should be opened with a prayer or with a sort of hoopla Perhaps the words of Aldous Huxley[2] should be quoted instead, "All great truths are obvious truths But not all obvious truths are great truths "

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1 Russell, B 1950 The Unpopular Essays Simon and Schuster New York, N Y
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Contents

Part III. Poliomyelitis Virus Variation

Part IV. Poliomyelitis Virus and the Community

Part V Immunization Against Poliomyelitis

INTRODUCTION

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arrogant, insufferable and vain" One feels this way only on rare occasions, and it is hoped that the results of this conference will prove how wrong and unjustified it would be to apply Valery's words to poliomyelitis research

Because of the subject of the conference, it is difficult to decide whether it should be opened with a prayer or with a sort of hoopla Perhaps the words of Aldous Huxley[2] should be quoted instead, "All great truths are obvious truths But not all obvious truths are great truths"

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Part I. Poliomyelitis Virus and Methods for Its Study

SOME PHYSICAL AND CHEMICAL PROPERTIES OF PURIFIED POLIOMYELITIS VIRUS PREPARATIONS*

By Carlton E. Schwerdt and Frederick L. Schaffer
Virus Laboratory, University of California, Berkeley, Calif.

Studies of the physical and chemical properties of the poliomyelitis viruses have progressed slowly in the past because of the difficulties encountered in the isolation of the virus particles in pure form from infected tissues.[1-6] Nevertheless, rough estimates have been made of the virus particle size by ultrafiltration and ultracentrifugation analyses of the biological activity of crude suspensions of virus.[7-11] The recent application of tissue culture methods to the propagation of the poliomyelitis viruses[12] has now greatly simplified the purification problem. Consequently, new attempts have been made to investigate the virus particle by more direct observations.[13-15] The purpose of this paper is to present the results of recent studies carried out in our laboratory on (1) the identification of type 2 (Lansing and MEF1) poliomyelitis virus from infected central nervous system (CNS) and tissue culture sources, and (2) some of the physical and chemical properties of the isolated and purified particles of MEF1 virus.

Identification of the virus particle. The identification of the virus particles depended in part upon their concentration in a highly purified state. Purification was achieved by a six-step procedure involving chemical and physical methods and is an outgrowth of the procedure developed earlier[16, 17] for the purification of Lansing virus from infected cotton rat CNS tissue.

The first five steps of the purification procedure used at present on poliomyelitis virus-infected monkey kidney tissue culture fluid (TCF) are outlined briefly below. Batches of TCF ranging from 1 to 30 liters are adjusted to pH 4 at 4°C. and chilled methanol (−50°C.) is added immediately thereafter to a final concentration of 15 per cent. The precipitated virus is filtered off on celite and eluted with 1 M NaCl at pH 9. The eluate that contains the virus is emulsified twice with n-butanol to extract lipids and denature nonviral proteins. Virus is then sedimented from the extracted eluate and clarified by one cycle of differential ultracentrifugation. The virus pellet resuspended in isotonic saline buffered at pH 7.5 with phosphate is treated at 37° C. for one hour with crystalline ribonuclease and desoxyribonuclease in final concentrations of 2 μg. of each per ml. It is then subjected to a final cycle of differential ultracentrifugation. The concentration of virus achieved at this point in the procedure may be 1000 to 40,000 times greater than that of the original TCF.

Purified virus concentrates prepared in this way from 30-liter batches of TCF contained a pigment which appeared to be absent from previous concen-

* Aided by a grant from the National Foundation For Infantile Paralysis, New York, N. Y.
The authors gratefully acknowledge the advice and aid of Doctor Howard K. Schachman in the electrophesis and sedimentation rate studies and of Doctor Robley C. Williams in the electron microscopy of the purified MEF1 virus preparations.

trates purified from one to eight liters of TCF Pigmented concentrates were subjected, therefore, to electrophoresis in a small Tiselius cell. In M/15 phosphate buffer at pH 7, the colored impurities moved as two components toward the anode with mobilities of approximately -4 and -5×10^{-5} cm^2/volt sec The major component which usually constituted approximately 80 per cent of the total mass of material as deduced from the schlieren diagrams moved in the same direction with the low mobility of -0.5×10^{-5} cm^2/volt sec This difference in mobilities has made possible the separation of the colored material from at least one half of the major component with which infectivity has been found to be associated

In our earlier work on the purification of Lansing virus from infected cotton rat CNS tissue,[16, 17] infectivity was followed by assay in cotton rats and expressed as the number of 50 per cent lethal doses per ml More recently, the infectivity of MEF1 virus purified from infected TCF has been measured by the plaque technique[18] and expressed as the number of plaque-forming units (PFU) per ml The monkey kidney epithelial cell monolayer used in the plaque assay was prepared essentially by Youngner's method [19]

The identification of the physical particle with which infectivity is associated was accomplished by means of analytical electron microscopy [17] This method utilizes the spray droplet technique developed by Backus and Williams [20] It involves, first, the spraying of microdroplets of a mixture of virus concentrate and polystyrene latex (PSL) particles of known concentration upon a collodion covered specimen screen, next, drying in air, shadow casting with uranium, electron micrography, and, finally, counting directly the PSL reference particles and any other characteristic particles of the virus concentrate observed in individual droplet patterns If all particles of the sprayed suspension are randomly mixed, it is possible to calculate the concentration of the characteristic particles from the ratio of these particles and PSL reference particles Thus, the correlation between any characteristic particle and infectivity can be determined

FIGURE 1 illustrates an electron micrograph of a droplet pattern of a mixture of PSL particles and purified Lansing virus concentrate from infected cotton rat CNS tissue The visible components in the circular pattern are the PSL ref

ph

un

are more clearly demonstrated in the enlarged insert of FIGURE 1 The average diameter of the 37 mμ particle packed in linear arrays of 5 or more particles is 28 mμ The latter value is believed to represent the more reliable estimate of the true diameter of the Lansing virus particle

The large 28 mμ particles were found only in concentrates from infected CNS tissue The amorphous material and small particles (12 mμ) were demonstrated in concentrates from both normal and infected CNS tissue The relationship between the number of 28 mμ diameter particles in Lansing virus concentrates and infectivity units was found to be constant from preparation to preparation Expressed as the ratio of physical particles and LD_{50} in cotton



FIGURE 1 Spray droplet pattern of purified Lansing virus concentrate from cotton rat CNS at ×8900 magnification with ×28,000 insert which shows A, the 28 mμ virus particles, B, the 12 mμ particles, and C, the amorphous masses

rats, the average value 21,000 ± 4000 was obtained for 10 different preparations

Infectivity was shown not to be associated with the smaller particles (12 mμ) by means of ultracentrifuge separation cell experiments. In these experiments, the infectivity was sedimented through a filter paper barrier in an analytical ultracentrifuge cell, so that 99 per cent of the virus activity was collected in the outer compartment while only 1 per cent remained in the inner chamber The distribution of the 28 mμ particles corresponded precisely with that of the infectivity under these conditions The distribution of the 12 mμ particles, however, was 52 and 48 per cent for the outer and inner com-

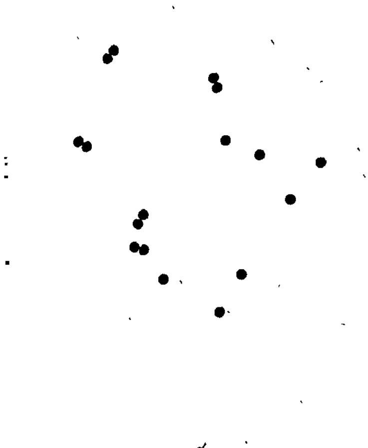


FIGURE 2 Spray droplet pattern of purified MEF1 virus concentrate from monkey kidney tissue culture fluid Magnification ×10,500

partments, respectively, which clearly indicated no correlation between the smaller particles and infectivity

The electron micrograph reproduced in FIGURE 2 is that of a typical spray droplet pattern of purified MEF1 virus concentrated from infected monkey kidney epithelial cell TCF There is no evidence of amorphous or small particle impurities in such a pattern The ratio of physical particles and PFU was observed to be approximately 1000 ± 500 Concentrates similarly prepared from either old and dying or young growing monkey kidney epithelial

TABLE 1
COMPARISON OF LANSING AND MEF1 PURIFIED VIRUS CONCENTRATES

	Lansing from CNS*	MEF1 from TCF†
Yield	*1 5 mg protein/kg*	*0 1 mg protein/l*
Specific infectivity	2×10^{-12} gm protein/LD_{50}**	$1 6 \times 10^{-14}$ gm protein/PFU‡
Physical particle/infective unit	$21,000 \pm 4000/LD_{50}$	1000 ± 500/PFU
Gm protein/physical particle	10×10^{-17}	$1 4 \times 10^{-17}$

* Lansing virus concentrate purified from infected cotton rat CNS
** Assayed by intracerebral inoculation in cotton rats
† MEF1 virus concentrate purified from infected monkey kidney TCF
‡ Plaque assay on monkey kidney epithelial cell layer

tissue-culture cells yielded no discrete, spherical particles visible by electron microscopy.

A comparison of the results of Lansing and MEF1 virus purification from cotton rat CNS and monkey kidney TCF, respectively, is summarized in TABLE 1. The best criterion of the relative purity of the type 2 virus particles from infected CNS and TCF is the ratio, grams protein per physical particle These data, recorded in the last row of TABLE 1, indicate that the tissue-culture source yielded purified concentrates of about seven times the purity of concentrates from CNS tissue.

Physical and chemical properties. The studies reported here on the physical and chemical properties of poliomyelitis virus are those concerned solely with MEF1 virus isolated in a concentrated and highly purified form from 10 to 30 liter batches of monkey kidney TCF. The properties determined by direct experimental observation are summarized in TABLE 2.

The external morphology of the virus particle was investigated by electron microscopy in collaboration with R. C. Williams and his associates [15] FIGURE 3 illustrates the spherical shape and uniform diameter of particles from an MEF1 virus concentrate The uniformity in this air-dried specimen is demonstrated by the two dimensional crystalline arrays of particles. The average diameter of the hexagonally packed particles is 27 mμ. Frozen dried specimens that are not subjected to the distorting effects of the surface tension forces of evaporating water yield individual spherical particles of the same diameter

The sedimentation of these spherical particles from an isotonic salt solution in an analytical ultracentrifuge is illustrated in FIGURE 4 The sharp boundary

TABLE 2
PROPERTIES OF MEF1 VIRUS PARTICLES DETERMINED BY DIRECT EXPERIMENTAL OBSERVATIONS

Diameter (electron microscopy)	27 ± 2 mμ
S_{20}	154×10^{-13}
Partial specific volume (sedimentation in D_2O)	64
OD_{260}/OD_{280}	1 69
$E_{1\,cm}^{1\%}$ at 260 mμ	67
Molar N/P	12 4
RNA fraction	.24
DNA fraction	< 01

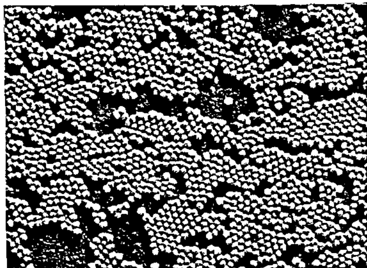


FIGURE 3 Purified MEF1 poliomyelitis virus air dried from an ammonium acetate suspension The uniformity of particle size is demonstrated by the existence of areas of hexagonal close packing Magnification ×73 000

seen in the sedimentation diagram confirms the particle homogeneity observed in the electron microscope Only one component is observed when either the ultraviolet absorption or the refractive index method of following sedimentation is used The sedimentation coefficient of a 0 02 per cent suspension of MEF1 virus particles is 154×10^{-3} cm /sec /dyne/gm

If protein or virus particles are sedimented in various water-deuterium oxide saline mixtures, and the products of viscosity and sedimentation coefficient in these mixtures are plotted as a function of the solvent mixture density, an estimate of the particles' reciprocal partial specific volume can be made by an extrapolation to zero sedimentation The validity of the relationship, particle density equals reciprocal partial specific volume, depends upon the



FIGURE 4 Sedimentation velocity of purified MEF1 poliomyelitis virus using ultraviolet absorption optics The mean centrifugal field for the first 15 exposures was 39 000 g that for the last 3 exposures, 260 000 g The interval between exposures was 2 minutes $S_{20} = 154 \times 10^{-13}$

assumption that the H_2O-D_2O salt mixture of both [illegible]

curve in FIGURE 5 Although the curve is fitted by the method of least squares, the extrapolation is quite long, and the estimated partial specific volume of 0 64 is subject, therefore, to some error. This determination must, of necessity, be repeated several times with different preparations of MEF1 virus particles before a precise value can be offered with confidence Nevertheless, the data obtained are sufficient to indicate that the particle is considerably heavier than simple protein, which has a partial specific volume of about 0 75

The ultraviolet absorption spectrum of a suspension of MEF1 virus particles shown in FIGURE 6 is one characteristic of a nucleoprotein with a maximum at 260 mμ and a minimum at 241 mμ. The ratio of optical densities at 260 and 280 mμ is 1 69 and the extinction coefficient at 260 mμ for a one per cent suspension is 67.

Chemical studies on MEF1 virus particle suspensions have been limited, so far, to an investigation of their nitrogen/phosphorus ratio and *nucleic acid* content. The particles' molar nitrogen/phosphorus ratio is 12 4 Their ribonucleic acid (RNA) content, as determined by the orcinol color reaction,[22] is 24 per cent, while the indole color reaction method of Ceriotti[23] suggests a desoxyribonucleic acid (DNA) content less than 1 per cent DNA, if present, therefore, is at a level within the limits of error of the method of analysis

Certain physical and chemical properties of the MEF1 particles have been estimated from related experimental data, as well as determined by direct measurements and analyses. For example, the partial specific volume of the particles has been calculated from the RNA fraction determined by chemical analysis In this instance, the assumption is made that the partial specific volumes of the protein and RNA moieties are additive. In addition, the diameter and mass of the particles have been estimated by means of Stokes's law, which applies to spherical particles of known sedimentation coefficient and partial specific volume Finally, the nucleic acid fraction has been roughly estimated from ultraviolet absorption data, nitrogen/phosphorus ratio, and partial specific volume measurements These estimates are summarized in TABLE 3

Discussion The following physical picture of the virus particle has evolved as the result of the data collected so far The particle is a uniform sphere with a diameter in the 22 to 27 mμ range Chemical analysis indicates it contains about 24 per cent RNA and has an estimated partial specific volume of 0 69 Physical measurement yields a partial specific volume figure of 0 64, from which a 52 per cent RNA content may be estimated The mass of the particle expressed in molecular weight units, calculated from high and low partial specific volume estimates, is $7\ 3 \times 10^6$ and $5\ 3 \times 10^6$ respectively

It was noted above (TABLE 1) that one plaque-forming unit was equivalent to approximately 1000 physical particles Dulbecco and Vogt[18] have shown, however, that the initiation of a single plaque is a one particle phenomenon Questions arise, then, regarding the other 99 9 per cent of the physical particles which form no plaques Are they inactive, or do the results mean that

the infection process is such that the probability of infection is 1 in 1000? If one considers them inactive, were they at any previous time infective, and do their chemical and physical properties differ from those which succeed in forming plaques?

We have found that, for any one preparation, the particle/plaque ratio is constant for the crude tissue-culture fluid and the purified concentrate. This suggests that if the purification procedure does inactivate the virus, it concomitantly causes particle disintegration. It is possible, however, to inactivate the virus thermally without parallel destruction of the particles themselves

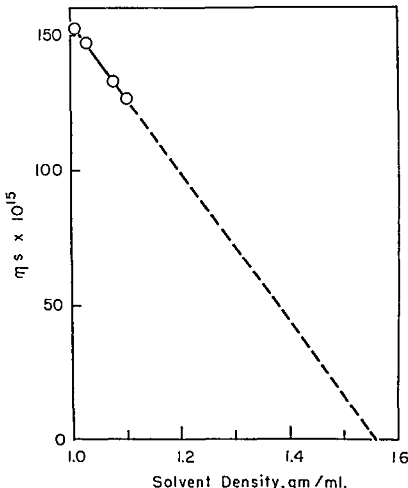


FIGURE 5. Sedimentation coefficients corrected for solvent viscosity of MEF1 virus particles in deuterium oxide aqueous saline solutions plotted as a function of solvent density. The straight line was fitted by the method of least squares.

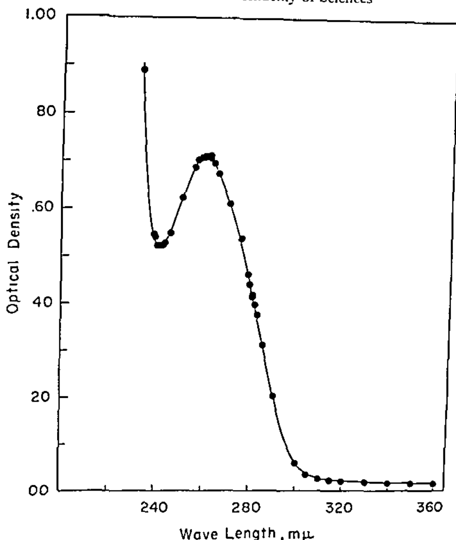


FIGURE 6. Ultraviolet absorption spectrum of purified MEF1 virus particles suspension in saline. Maximum at 260 mμ and minimum at 241 mμ.

Exposure to 37° C. for 66 hours results in 90 per cent loss of infectivity, but causes the destruction of only 10 per cent of the number of physical particles.

Studies are now being made to determine whether the high particle/plaque ratio is the result of assaying virus preparations in which approximately only one particle per 1000 is really viable or is due to the infection of the tissue culture under suboptimal conditions.

Summary. MEF1 poliomyelitis virus infectivity has been found to be associated with spherical particles, uniform in size, with a diameter in the 22 to 27 mμ range. Chemical and physical studies indicate that the particles are

TABLE 3

PROPERTIES OF MEF1 VIRUS PARTICLES ESTIMATED FROM RELATED EXPERIMENTAL DATA

Property	Experimental		Estimation	
	Observation	Method	Value	Method
Partial specific volume, $\bar{V}$	0 64	Sedimentation in D_2O H_2O mixtures	0 69	RNA analysis
Diameter (mμ)	27	Electron microscopy	22	Stokes' law, $\bar{V}$ = 0 64
			25	Stokes' law, $\bar{V}$ = 0 69
Mass (gm)	$1\ 4 \times 10^{-17}$	Protein analyses and electron microscope count	9×10^{-17}	Stokes' law, $\bar{V}$ = 0 64
			$1\ 2 \times 10^{-17}$	Stokes' law, $\bar{V}$ = 0 69
RNA fraction	24	Orcinol color reaction	22	$I^{1\%}_{1\,cm}$ at 260 mμ
			30	Nitrogen/Phosphorus
			.52	$\bar{V}$ = 0 64

nucleoproteins of the RNA type, that the nucleic acid constitutes approximately one fourth to one third the mass of the particles, and that they have a low partial specific volume compatible with their chemical composition The physical particle/plaque ratio is approximately 1000, for which an explanation has yet to be found

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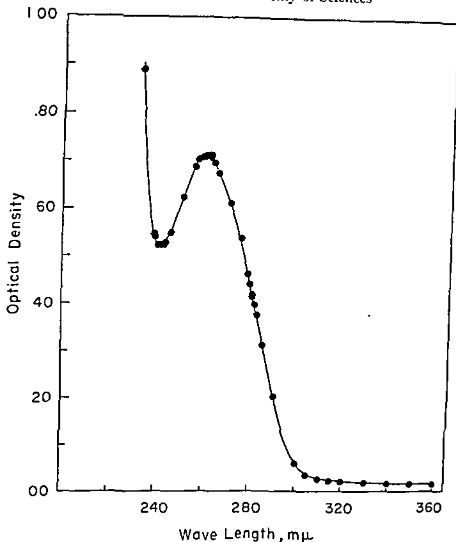


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Discussion of the Paper

DOCTOR A. R. TAYLOR (*Parke, Davis & Co., Detroit, Mich.*): We are in essential agreement with Doctor Schwerdt and his co-workers on the general

types ... form of purified concentrates from filtered, trypsinized monkey kidney cell tissue-culture fluids (titer 10^{-8} TCD per ml.), by means of a combination of alcohol

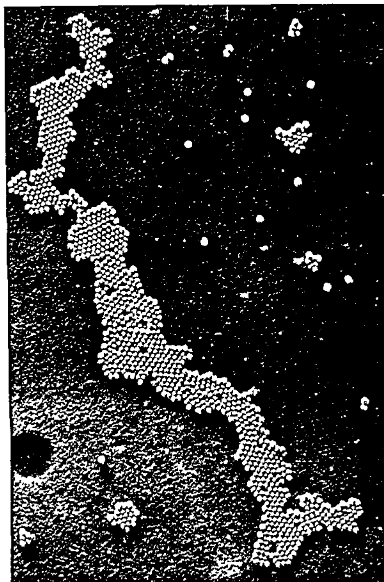


FIGURE 1. Poliomyelitis virus (MEF₁) collodion over agar filtration mount shadowed with chromium after dehydration by allowing prolonged contact of the collodion membrane with the agar. Particles are flattened and the apparent diameter measures 32 mμ (internal calibration polystyrene latex). ×53000

FIGURE 2. Poliomyelitis virus (MEF1) shadowed and un... smaller than the shadowed image and the electron opaque mat... centrally. Particles are also variable in size and shape. X63

so that the particles are hydrated at the time of the first exposure to high vacuum, the spherical shape is readily demonstrated In preparations dehydrated before shadowing, however, we find definite flattening and increase in the apparent diameter, sometimes as much as 15 per cent (FIGURE 1). We believe also that there is evidence of compression of the particles visible in the central areas of large, closely packed arrays Comparison of shadowed and unshadowed images of the virus (FIGURE 2) reveals that the transmission image is definitely smaller than the shadowed one, even when the thickness of the shadowing metal is considered This is due apparently to the presence within the particle of dense electron opaque material It is of interest to note that the transmission images are quite variable in size In clumps and closely packed arrays, there appears to be an indication of a distinct spacing around the dense central areas This spacing is evident in preparations made both from saline media and also from salt-free media

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TISSUE CULTURE TECHNIQUES AND THEIR APPLICATION TO ORIGINAL ISOLATION, GROWTH, AND ASSAY OF POLIOMYELITIS AND ORPHAN VIRUSES*

By Joseph L. Melnick

Yale University School of Medicine, New Haven, Conn

The first successful cultivation of poliovirus† in tissue culture was accomplished almost 20 years ago by the use of human embryonic nervous tissue. However, it was the discovery in 1949 by Enders, Weller, and Robbins, that poliovirus can multiply in cultures of nonnervous tissue which led to the present revolution in methodology of virus research. I dare say that most of the data to be discussed in this monograph will have been obtained by the newer tissue-culture methods, for these *in vitro* methods have proved to be faster, more convenient, and more accurate than methods involving the inoculation of monkeys or mice. Techniques have been developed for the isolation and antigenic identification of polioviruses from clinical materials, and for the quantitative measurement of neutralizing antibodies. Furthermore, through the use of tissue cultures, complement-fixing antigens of all three viral types have become available.

At the present time, details of procedure vary from one laboratory to the next, as may be expected during a stage of rapid evolution. The choice of methods is often dictated by local supply of suitable tissues and other materials, although recent success with shipping susceptible cells from one continent to another has made the local availability of tissue less of a problem. A

tionary-tube cultures with tissue fragments made to adhere to the glass surface by preheating the vessel,[6] and susceptibility of cultures prepared from immunized animals.[7] These methods will not be discussed here, nor will the HeLa culture technique be described, for this method has been reviewed in detail by Syverton and Scherer.[8] Rather, I shall bring to your attention certain more recently developed procedures and attendant problems. The present discussion will deal with (1) methods of preparing trypsinized suspensions of monkey kidney cells and their subsequent growth in cultures, (2) multiplication of virus in such cultures, and (3) the use of tissue cultures in the isolation of poliomyelitis and orphan viruses from clinical specimens.

Part I Recent Developments in Tissue Culture Techniques

Dulbecco and Vogt[9] have reintroduced the Rous-Jones trypsinization procedure for obtaining cells directly from minced tissue and growing them on glass

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc., New York, N. Y.

† The term *poliovirus* is used here upon the recommendation of the Virus Subcommittee of the International Nomenclature Committee of the International Congress of Microbiology. The binomial of the virus of human poliomyelitis is given as *Poliovirus hominis*.

surfaces in the absence of a plasma clot Cell suspensions prepared from kidney tissue produce luxuriant monolayer cultures on any clean glass surface in such containers as Petri dishes, test tubes, or bottles held in the stationary position The method has been improved and made more quantitative by Youngner[10] More recently, Catherine Rappaport,[11] working in New Haven, has been systematically investigating the procedure, with the result that the yield of cells per kidney has been increased by two to three times, and the cell suspension stabilized so that it can withstand shipping long distances, as from Bombay, India, to New Haven, Conn

The revised procedure uses a specially designed flask (FIGURE 1) which permits the use of a magnetic stirrer and has proved to be more convenient than the Waring blendor. The sides of a 500-ml Erlenmeyer flask have been indented to increase the turbulence and cavitation during stirring, and a glass filter and outflow tube have been built into the bottom of the flask. After a preliminary incubation of one hour with prewarmed trypsin, warm trypsin is allowed to flow dropwise through the mixing chamber, exhausting the kidney fragments of their cells in a few hours Trypsin is led in from a reservoir The entire operation is automatic and takes place in a closed system The trypsinization fluids flow into a bottle held in an ice-water bath, and the cells are collected by centrifuging at 200 rpm (11 times gravity) for 30 minutes The force of centrifugation is critical, for higher speeds, even for short periods, have been found to destroy 30 to 60 per cent of the cells About three ml of packed cells are obtained per kidney The trypsin is removed by aspiration, and the cells are resuspended in prewarmed growth medium An average yield of 4 to 5 $\times$ 10^9 cells are obtained per kidney

Rappaport found that freshly trypsinized kidney cells liberate a heat labile cytotoxic material, to the detriment of the culture Thus, as shown in FIGURE 2, within hours after the cells are held at 37° C, the cell count begins to drop, reaching a minimum 24 hours later with only about 10 per cent of cells remaining, a finding which agrees with Youngner's recent report[10] Concomitantly with the decrease in the cell population, the cytotoxin increases in concentration The amount formed is a function of the number of cells and the result of active metabolism It is not produced in measurable amounts in media that do not support cellular growth and multiplication In such deficient media, no cytotoxin is produced, even though cellular disintegration may be complete in 12 hours

The cytotoxin is readily assayed by its action on 24-hour cultures, for it lyses the cells of such cultures in an hour or two Cytotoxin is produced spontaneously in young cultures, but not in cultures which are about a week old and have grown into sheets of epithelial cells If inoculated into old cultures, however, cytotoxin is produced and can be carried in series The material is inactivated by heating at 60° C for 30 minutes

We have not determined whether cytotoxin is synthesized normally in cells and liberated by cytotoxin-induced lysis, or whether cytotoxin actually induces its own synthesis, as in the case of the classic infectious agents Inoculation of the cytotoxin into monkey kidneys *in vivo* failed to produce any disease and,

TISSUE CULTURE TECHNIQUES AND THEIR APPLICATION TO ORIGINAL ISOLATION, GROWTH, AND ASSAY OF POLIOMYELITIS AND ORPHAN VIRUSES*

By Joseph L. Melnick
Yale University School of Medicine, New Haven, Conn.

The first successful cultivation of poliovirus† in tissue culture was accomplished almost 20 years ago by the use of human embryonic nervous tissue. However, it was the discovery in 1949 by Enders, Weller, and Robbins, that poliovirus can multiply in cultures of nonnervous tissue which led to the present revolution in methodology of virus research. I dare say that most of the data to be discussed in this monograph will have been obtained by the newer tissue-culture methods, for these *in vitro* methods have proved to be faster, more convenient, and more accurate than methods involving the inoculation of monkeys or mice. Techniques have been developed for the isolation and antigenic identification of polioviruses from clinical materials, and for the quantitative measurement of neutralizing antibodies. Furthermore, through the use of tissue cultures, complement-fixing antigens of all three viral types have become available.

At the present time, details of procedure vary from one laboratory to the next, as may be expected during a stage of rapid evolution. The choice of methods is often dictated by local supply of suitable tissues and other materials, although recent success with shipping susceptible cells from one continent to another has made the local availability of tissue less of a problem. A number of articles[1-3] have reviewed the early phases of tissue culture methodology that dealt with cultures of suspended fragments of tissues,[4] roller-tube cultures with tissue fragments fixed to the vessel wall by a plasma clot,[5] stationary-tube cultures with tissue fragments made to adhere to the glass surface by preheating the vessel,[6] and susceptibility of cultures prepared from immunized animals.[7] These methods will not be discussed here, nor will the HeLa culture technique be described, for this method has been reviewed in detail by Syverton and Scherer.[8] Rather, I shall bring to your attention certain more recently developed procedures and attendant problems. The present discussion will deal with: (1) methods of preparing trypsinized suspensions of monkey kidney cells and their subsequent growth in cultures, (2) multiplication of virus in such cultures, and (3) the use of tissue cultures in the isolation of poliomyelitis and orphan viruses from clinical specimens.

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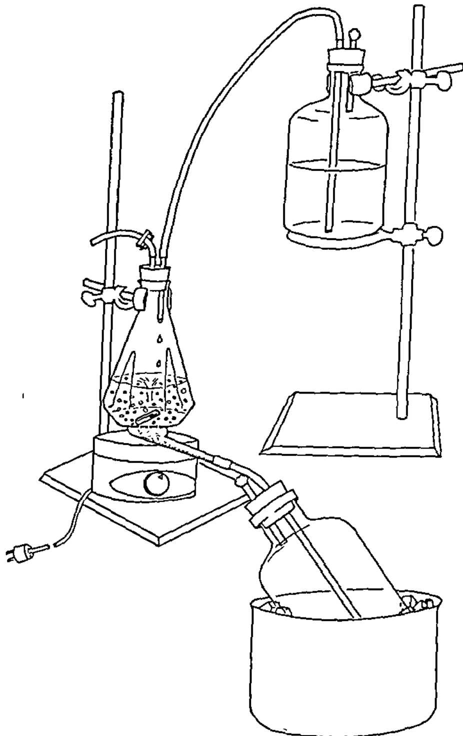


FIGURE 1 Apparatus for the automatic trypsinization of monkey kidney Warm trypsin is siphoned into the flask in which mixing takes place by means of a magnetic stirrer The turning of the stirrer keeps the tissue fragments from clogging the pores in the glass filter built into the bottom of the flask The cell suspension flows into a reservoir held in an ice water bath

a few weeks after inoculation, the kidneys of the animals were removed and

to those which contain complex synthetic mixtures such as No 199 fortified by serum or proteins derived from it [12] For use with trypsinized cells, we have developed an inexpensive medium which is simple to prepare It consists of 0 5 per cent lactalbumin enzymatic hydrolysate, 2 per cent calf serum, and

0 5 ml The tubes are then held stationary at a slight incline at 37° C. In about four days, epithelial sheets form and the pH begins to fall At this time, the culture fluid is replaced with one ml of the lactalbumin medium,

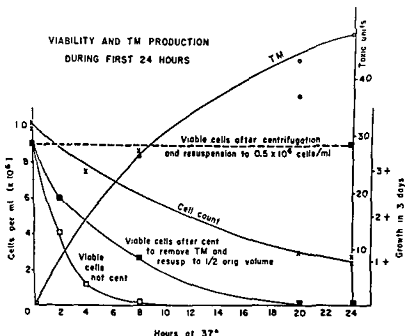


FIGURE 2 Decrease in cell count and

in which the more highly buffered Earle's solution is substituted for Hanks'. Such cultures have a useful lifespan of about two weeks, without any further change of the fluid phase.

Youngner has reported that the viability of cell suspensions is retained at a usable level for at least two days beyond the day of preparation, when kept at 4° C. in medium 199 to which 2 per cent horse serum has been added. In our experience, we have found that cells retain their viability, with little loss in number, for at least five days when kept at 0° C. in our 0.5 per cent lactalbumin hydrolysate medium to which 4 per cent calf serum has been added. Some suspensions have produced useful cultures after storage for over two weeks. This has enabled us to set up a cooperative experiment with Doctors Dushyant Banker and Pravin Bhatt of the Seth G. S Medical College in Bombay, India. Using methods that were outlined to them in correspondence, these workers have been sending us weekly shipments of trypsinized cells. The refrigerated suspensions have been sent by air, arriving in the United States two days after being dispatched from Bombay. As shown in TABLE 1, the cell counts in New Haven do not vary markedly from those made on the suspensions before leaving Bombay. In New Haven, the cells have produced satisfactory culture tubes and bottles, which have responded to poliovirus as quantitatively, and have produced complement-fixation antigens as readily as cultures made from locally prepared cell suspensions.

Although there have been no reports as yet on the serial propagation of monkey kidney cells *in vitro*, efforts have been made in this direction, and I know that we have not been alone in these efforts. It is not difficult to carry kidney cells for two or even three serial transfers but in our hands, beyond this

TABLE 1

UTILIZATION IN NEW HAVEN OF CELL SUSPENSIONS PREPARED IN BOMBAY

Lot number	Cell count in millions per ml		Days* of storage at 0°C	Days† of incubation
	In Bombay	In New Haven		
4	0.6	0.6	4	5
4	0.6	0.6	8	5
4	0.6		11	6
5	0.6	0.3	4	5
5	0.6	0.2	8	6
6A	0.6	0.3	5	5
6B	1.2	0.9	5	5
6C	2.4	1.7	5	5
6C	2.4	0.6	9	7
7A	0.6	0.5	5	7
7B	1.2	1.0	5	7
7C	2.4	2.2	5	5
8A	0.6	0.5	5	5
8B	1.2	0.9	5	5
8C	2.4	1.7	5	5

* Days after trypsinization that cells were stored, including the time of transit on ice. Cells were recounted on the day they were used for seeding cultures. From 0.2 to 0.3 × 10^6 cells were used to seed culture tubes, and 1.5 to 2.0 × 10[illegible] cells for Roux bottles.

† Days of incubation necessary to form epithelial sheet after cultures were seeded.

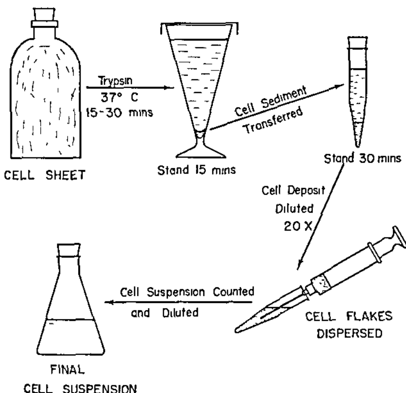


FIGURE 3 Preparation of suspension of second or third transfer cells from culture of monkey kidney grown in a Roux flask The cells come loose as small flakes and quickly settle so that centrifugation is unnecessary

point, cultures have quickly lost their viability Even so, second transfer kidney cells offer certain advantages for the virus laboratory, as already shown by Dulbecco and Vogt

They may readily be prepared[11] as illustrated in FIGURE 3 A one-week-old primary culture in a Roux flask contains about 10^7 cells The fluid phase of such a culture is replaced with 40 ml of 0 25 per cent trypsin in phosphate-buffered saline (prewarmed to 37° C) Within 15 to 30 minutes, the epithelial sheet comes loose as small flakes The trypsin, plus flakes from a number of bottles, is poured into a conical vessel and allowed to stand until the flakes settle to the bottom (about 15 minutes) The bulk of the trypsin solution is removed and the flakes are transferred to a conical graduated centrifuge tube and allowed to settle for 30 minutes This permits an accurate measurement of cell volume and also the removal of most of the trypsin solution Twenty volumes of lactalbumin-calf serum medium are added, and the flakes dispersed by drawing into, and ejecting from, a pipette or a 20-ml syringe equipped with

an 18-gauge needle 4 or 5 times, or until the appearance of the suspension suggests that few large cell clumps remain. An aliquot of the cell suspension is stained with two volumes of 0 1 per cent crystal violet in 0.1 M. citric acid, and counted in a hemocytometer For use in seeding cultures where an epithelial sheet is desired in 24 hours, the suspension is diluted to give 150,000 cells per 0 5 ml, the volume used for seeding. For use in the colorimetric test of Salk, Ward, and Youngner,[13] 40,000 cells per 0 25 ml. volume are sufficient for addition to each tube.*

Second-passage cells have a greater viability than primary cells and, because little loss occurs during the first day of incubation, they can be employed at smaller concentrations One further advantage is that second-passage cells may be stored in the lactalbumin-calf serum medium at 4° C. for several days w [illegible]

I [illegible]

been made. First, Chang[14] has grown epithelial cells derived from *normal* human tissues (conjunctiva, liver, kidney, appendix), and has made continuous subcultures by trypsinizing the parent cultures and cultivating the subcultures in human serum The cell lines proved to be susceptible to poliovirus

Second, Scherer and Hoogasian[15] found that HeLa cells may be preserved for at least seven months at −60° to −70° C if 20 per cent glycerol is added to the suspending medium Thus, the cell line may be stored frozen when it is not needed in the laboratory and, in addition, cells of the same passage level may be obtained for use in different experiments.

Part 2 Growth of Established Strains of Poliovirus in Tissue Culture

Poliovirus grows in susceptible cells in fashion similar to the multiplication of phage in bacteria First, the virus particles are adsorbed to the cells A latent period follows in which the amount of virus in the culture fluid remains constant for a few hours, and then new virus particles are released from the infected cells If the number of virus particles inoculated is insufficient to infect all the cells in the culture, the process is repeated until all the susceptible cells are infected Regardless of the size of the inoculum, the total amount of virus produced by the cultures having the same number of cells is the same This is illustrated in FIGURE 4 Two sets of identical cultures were inoculated with 10^2 and 10^5 TC_{50} doses of Brunhilde virus The cultures receiving the large dose produced their maximum yield of virus in 24 hours, while 48 hours were required for the second set of cultures Both sets of cultures, however, produced the same amount of virus, which failed to increase after the second day

Others in this monograph will discuss the one-step growth curve of poliovirus

* [illegible] cells may also be prepared without the use of trypsin A chelating agent sodium versenate is used [illegible]

[illegible] tion is necessary

in tissue culture cells—even in a single isolated cell—where the plaque technique has been applied with great success. I should merely point out here that data obtained by Howes and Reissig on the WS type I strain, shown in FIGURE 5, indicate that intracellular virus may not leave the cell as soon as it is formed.[11] After the adsorption and lag phases, new virus made its appearance. For a period of a few hours, however, the amount of new virus associated with infected cells was greater than that found free in the extracellular fluid.

As is well known, poliovirus is now being produced in monkey kidney cultures in quantities sufficiently large for the commercial production of a vaccine. It is not my purpose, however, to discuss any such large-scale production of virus. Papers from Rhodes' and Salk's laboratories[16, 12] have recently appeared, dealing with the growth of virus in bulk cultures of suspended tissue fragments or of trypsinized cells. For the production of virus in amounts sufficient for most university laboratories, cultures in one-liter Roux flasks, or other flat-sided bottles of similar size, are recommended. As Miller[17] has shown, they are convenient to manage, and, when used for kidney monolayer cultures, the 10^7 cells that they each contain can be infected and made to release their virus into a volume of 30 ml.

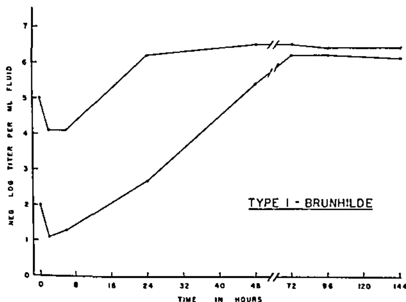


FIGURE 4. Yield of virus from cultures having the same number of cells but inoculated with two doses of virus differing by 1000 fold in concentration. The total amount of virus produced was the same in each culture.

important leads for future investigation. As an example, the data in TABLE 2 show the differences in growth found in New Haven when monkey kidney adapted viruses were titrated in kidney and in HeLa cultures. [illegible]

[illegible] experiments were obtained from [illegible]

thesda, Md., a point [illegible]

Morphological cha[illegible]

exerts a destructive action on the cells. Indeed, it was this cytopathogenic activity of the virus which led Enders *et al.* to develop the first rapid method for titrating poliovirus. From their experiments with fluorophenylalanine, Ackermann *et al.*[15] hold that, in HeLa cells at least, the cytopathogenic action of the virus can occur independently of viral multiplication. This is almost the reverse of the situation which we observed when Lansing virus multiplied in monkey testicular fibroblasts with little evidence of cytopathogenicity.[19] It should be pointed out that this line of Lansing virus has shown other evidence of low virulence for nonneural tissues. In contrast to the homotypic Y-SK strain, it failed to induce antibody formation in orally infected cynomolgus monkeys.[20] The Lansing strain has also been shown to differ from other homotypic strains (Y-SK and MEF_1) by its greater thermolability.[33]

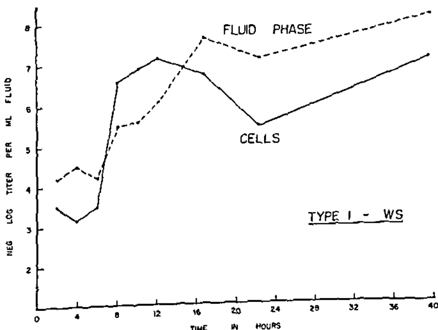


FIGURE 5. Concentration of virus in cells and in the extracellular fluid phase after introduction of virus into the culture. The cells were washed, disrupted, and then diluted to the same volume as the fluid phase of the culture. Virus appears to be associated with cells in high titer before [illegible] in concentration in the fluid phase. Data obtained by David Howes.

TABLE 2

ADAPTATION OF MONKEY KIDNEY PASSAGED VIRUS TO HELA CULTURES

Type	Strain	Passages in monkey TC	Passages in HeLa	Titer* of TC fluid in	
				HeLa	Monkey kidney
I	WS	141	0	4 8	6 5
			4	5 5	7 0
II	Y-SK	145	0	4 0	6 5
			4	6 0	7 0
III	Leon	131	0	5 0	8 0
			4	6 5	6 3

* Negative logarithm of titer per ml

Usually, cytopathic changes can be correlated with the extent of virus growth. The photomicrographs in FIGURES 6 through 9, taken by Doctor Magdalena Reissig under the phase microscope, show the evolution of the lesion Swelling of the cells begins a few hours after virus inoculation, and this swelling is accompanied by marked vacuolization of the cytoplasm and shrinkage of the nucleus There is apparent condensation of basophilic material about the inner surface of the nuclear membrane Doctor Reissig has found that one or more eosinophilic inclusion bodies are usually present in the nucleus at this stage Later in the infection, the cells become rounded, acquiring an appearance which characterizes the cytopathogenic effect of poliovirus in tissue cultures, as usually observed At this stage, large vacuoles are present in the cytoplasm As shown in FIGURE 9, they are readily seen in living cells under the phase microscope, as well as in stained preparations A marked increase in the basophilia of the peripheral zone of the cytoplasm is also observed at this time Nucleoli persist until the late stages, when the nuclei become markedly pyknotic and eccentric and their internal structure can no longer be distinguished Ultrathin sections of such cells have been prepared by a method recently described[71] and are being studied in the electron microscope, but the results are too preliminary to warrant discussion here

Part 3 Assay of Virus

With the exception of the elegant plaque assay method, which will be dis-

cultures become infected to one where none becomes infected The end points are determined by the method of Reed-Muench or, more simply and with no loss in accuracy, by the method of Kaerber By increasing the number of cultures at each dilution, the error of the titration end point is reduced A convenient number which gives reasonably reproducible results is four cultures at each half log dilution As different workers may use different volumes of inoculum in their titration series, it would make for uniformity if all expressed their results in the same fashion namely as the titer per ml of original fluid.

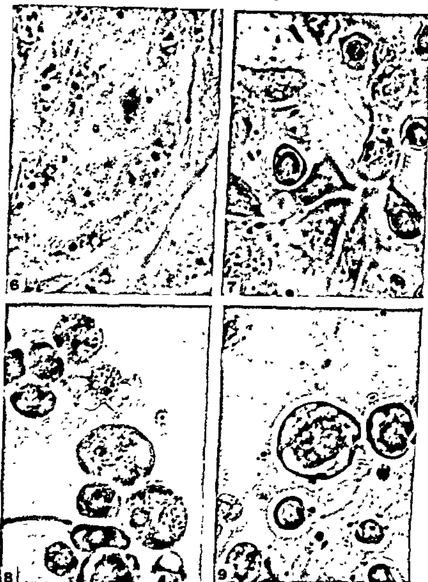


FIGURE 6. Cells from a monolayer culture of monkey kidney epithelium. Uninoculated control. ×410. This photomicrograph, as well as those in FIGURES 7, 8, and 9, were taken under the phase microscope by Magdalena Reissig.

[illegible] nucleus. ×330.

Another variable which occurs from one laboratory to the next is the use of animal sera in culture fluids Many animal sera may inhibit the growth of poliovirus, and care must be exercised that such sera are not present in the culture when virus is added for assay

The methods of assaying infectivity are based on the quantitative measurement of the destruction of cells by poliovirus, as outlined in the classical paper published in 1950 by Robbins, Enders, and Weller [22] In the cytopathogenic test, the destruction of cells is followed microscopically In the pH-differential test, use is made of the fact that infected cells fail to maintain their metabolic rate. Metabolism is measured by acid production, and a change in color of the phenol red indicator from red to yellow occurs in normal cultures, but not in infected cultures Salk, Youngner, and Ward[13] have shown that this color test can be used with monkey kidney cell suspensions containing a known amount of cells to permit a much higher degree of quantitation, and others have since adapted the color test for virus assay, using suspensions of HeLa cells

The color test[13] is being used in a number of laboratories where it is proving to be a tremendous time saver Because it is discussed in detail in connection with the neutralization test elsewhere in this monograph, I shall mention only the slight modifications of the technique now in use in New Haven

First, we have been able to carry out the tests without the complex medium 199 The lactalbumin hydrolysate-calf serum medium has proved a simple and satisfactory replacement

Second, if second-passage monkey kidney cells are used, as discussed earlier in this paper, the concentration of cells per culture tube may be reduced from 300,000 to 50,000, or even less

Third, the disposable plastic panel that McLean[14] introduced into the poliomyelitis laboratory can be employed here to advantage This panel, made of a styrene or vinyl plastic, contains 96 cups in rows of 8 × 12 It is clean and ready for use as it comes from the manufacturer † Sterilization is easily accomplished by immersion in an alcohol bath and exposure to ultraviolet light The use of plastic panels eliminates the need not only for test tubes but also for rubber stoppers, for the panel cups may be sealed in rows with coated celloph[illegible]pe Cells settle and grow on the plastic as well as [illegible] test is read, the panels are autoclaved and dis- [illegible] has been added to the cups, metabolism [illegible] pH falls before virus has multiplied [illegible] can readily be obtained with the [illegible]n, the cellophane sheet over each [illegible]O_2 allowed to escape so that the [illegible]led with cellophane tape, and [illegible]livision between yellow cups [illegible]ls killed by virus action

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McLean[31] has found that the virus-containing cultures may be "sealed" by a layer of mineral oil and this finding has been confirmed by Opton[11] Be-

† Linbro Chemical Co New Haven Conn

cause CO_2 passes slowly through the oil, phenol red will turn yellow only when cellular metabolism is vigorous and the rate of CO_2 production exceeds that of CO_2 loss. If the cells are in excess, so that they grow faster than the inoculated virus can destroy them, the phenol red will turn yellow. If virus is present, however, it multiplies to such a level in a few days that all cells are killed. The CO_2 in the culture is now in negative balance, and more does not form to replace that which leaks through the oil. This is indicated by the color of the culture, which reverts to red.*

Part 4. Isolation of Virus

Methods for the isolation of poliovirus may differ in detail from one laboratory to another, but the same basis underlies them all, namely, after the addition of the clinical specimen to the tissue culture, there is liberated into the [illegible] inactivation of bacteria is required before such samples can be inoculated into tissue cultures. The problem of cleaning up the specimen is not markedly different from that which we faced when intracerebral inoculation of monkeys was the method of choice, and the procedures worked out for monkeys[23] have been applied to preparing specimens for tissue culture inoculation. The procedures for virus isolation can be made more sensitive by concentrating the virus present by ultracentrifugation, which at the same time frees it from offending bacteria and certain other toxic substances. For routine purposes of isolation of virus from patients or carriers, it is sufficient to make a 10 to 20 per cent suspension of stools, centrifuge at 3000 rpm for 60 minutes, and add antibiotics (penicillin, 500 units, and streptomycin, 0.5 mg. per ml.) to the supernatant fluid before inoculation of the cultures. Syverton and Scherer[5] recommend suspending the stools in water containing 20 mg. phenol red per liter to avoid specimens with abnormal and cytotoxic hydrogen ion concentrations.

Comparative studies have demonstrated that epithelial monolayer cultures from monkey kidneys are more sensitive for primary isolation of virus than suspended fragments of kidney tissue or of fibroblast cultures from monkey testis.[35, 36] Another advantage of kidney epithelial cells is their greater resistance to fecal toxins. In addition to our studies, Wenner and Miller[37] have reported a series of experiments in which, for primary isolation of poliovirus, monkey kidney epithelial cultures proved superior to testicular cultures and also to HeLa cultures with which they were compared. Other papers in this monograph refer to the comparative susceptibility of human and monkey cells.

Studies of materials collected from the same patients and family contacts have shown that poliovirus can be isolated at about twice the frequency from

* Salk reported at the conference on which this monograph is based that tubes may also be sealed with oil rather than rubber stoppers. He speculated that the reversion of the culture from yellow to red was the result of the formation of an alkaline substance by the virus infected cells.

stools as from rectal swabs. While many individuals yielded positive stools but negative swabs, the reverse situation was a rare occurrence

I shall not take time here to present the comparative experiments which led to the procedure which we now use Suffice it to say that the highest percentage of virus isolations were obtained with a procedure which, more or less, is like that which Enders first recommended.

Five culture tubes are used for each specimen The fluid phase is removed and replaced with one ml of the stool extract. Adsorption of the virus is allowed to proceed for 90 minutes at 37° C., after which the fecal extract is removed and replaced with 1 ml of fresh lactalbumin hydrolysate medium in Earle's solution The cultures are observed on the 2nd, 4th, 7th and 10th days for cytopathic changes induced by the presence of the virus Fluid is harvested when these changes appear, and it is tested for the presence of poliovirus by its reaction with type-specific antisera

Typing by immune sera of any cytopathogenic agent which may be isolated is essential for its identification This may be done by neutralization or by complement-fixation tests For complete characterization of an agent, box titrations may be necessary, but this is not a practical typing procedure, particularly in the neutralization test A procedure which gives satisfactory results is the following:

A 1:10 dilution of fluid harvested from cultures manifesting cytopathic changes provides the virus for typing Type-specific antisera are used at a 1:5 dilution These sera, prepared by hyperimmunization of monkeys, should have titers of over 1:1000 against 100 TC_{50} doses of virus To 0 2 ml of each serum dilution in separate tubes is added an equal quantity of the virus dilution The tubes are shaken, incubated for one hour at room temperature, and 0 1 ml of each mixture inoculated into two culture tubes The cultures are incubated at 37° C in a stationary position until read on the third and sixth days The virus is identified in accordance with the type-specific serum which neutralizes its cytopathogenic property If a virus is not neutralized by any of the three antisera, it must be tested further before being placed with the orphan viruses[24] High titer poliovirus may break through its homologous antiserum or, in very rare instances, two poliovirus types may be present in the same specimen Therefore, the virus must be tested in small doses against the three prototype sera alone and in combination If it is still not neutralized, then it may be placed tentatively with the orphans (see *Part 5* below) until identified further

Isolation and typing may be accomplished in one step, and this procedure is the most rapid for typing viruses in clinical specimens The stool suspension is mixed with equal volumes of 1:10 dilutions of each of the three prototype antisera, and after a one-hour incubation period, 0 2 ml of each mixture is then inoculated into each of two or more culture tubes The virus type is identified at the time of its primary isolation, for degeneration is observed in the tubes containing the heterotypic sera, but not in the tubes containing the homotypic serum By this technique, virus has at times been isolated and typed within 48 hours

DISTRIBUTION OF TITERS

	TITER	TYPE I	TYPE II	TYPE III
TYPE I ANTIGEN	240	ooooooooooo OO		
	120	ooooooooooooooooooo OOOOO	o O	O
	60	oooo	o O	
	30		oooo OOO	o
	15		ooooooooooo OO	
	±		ooooooooo	
	0		oooooooo	ooooooooooooooooo oooooooooooooooooo OOOOOO
TYPE III ANTIGEN (SERUM TITER)	240			oo O
	120			oooooooooooooo O
	60		O	O
	30		oooooo	
	15	o O	ooooo OO	
	±	o	o	
	0	oooooooooooooo OO	oooo	

KEY: o NEW ENGLAND; O CAIRO

[illegible] of C F titers of three standard monkey immune sera tested against 34 type I strains [illegible]

Polioviruses may also be typed by employing them as antigens, at concentrations of four to eight units, in the complement-fixation reaction against standardized prototype antisera[25] By using the plate technique, in which [illegible]

c

n

to strain when tested with monkey immune sera shown to be homotypic with prototype Brunhilde, Y-SK, and Leon strains As shown in FIGURE 10, certain type I strains failed to react with type II or type III serum, while others exhibited some crossings with Type II serum, and two with both types II and III at the serum dilutions indicated In similar fashion, type III strains were either monotypic or showed crossings with type II serum.

Part 5. Orphan Viruses

Mention has been made of cytopathogenic agents other than poliovirus that may be isolated by tissue-culture techniques. Because this is the only paper in this monograph dealing specifically with problems of poliovirus isolation, I shall say a few words about these agents, which have been encountered unexpectedly in these procedures. Because we know so little about the diseases to which they belong, we have provisionally called them *orphan viruses*.[26] These agents have been isolated both at home and abroad. In this country, they have been isolated chiefly from the stools of patients with

of aseptic meningitis, enteritis, hepatitis, and perhaps even type IV poliovirus. Certain orphan viruses have been found to belong to the Coxsackie group, by serologic identification or when the fluids of infected cultures are tested in

produced serum which neutralized the tissue culture agent at a passage stage when it was without pathogenicity for mice. Similarly, the early tissue culture passaged material produced an immune serum in the monkey that neutralized the murine agent subsequently derived from the tissue culture virus.

In any case, as shown in TABLE 3, the orphan viruses fall into at least four antigenically distinct types. The prototype strains are not neutralized by any of the three poliomyelitis antisera or the 15 Coxsackie immune sera against which they have been tested. Complement-fixing antigens are released into tissue culture fluids when the orphan viruses are cultivated in monkey-kidney epithelium. When tested against monkey-immune sera, the reactions were as specific for each type as found in the neutralization tests shown in the table.

The sizes of the prototype orphan viruses have been measured by ultrafil

TABLE 3
CROSS NEUTRALIZATION TESTS WITH ORPHAN VIRUSES

Virus	Immune Serum Neutralization Index					
	Control Titer*	Orphan type I	Orphan type II	Orphan type III	Coxsackie type A 9	Polio type I
Orphan type I (Farouk)	5.0	1000+	0	0	0	0
Orphan type II (Cornelis)	4.5	3	1000+	0	0	0
Orphan type III (Morrisey)	4.8	0	3	1000+	0	0
Orphan type III (Rende)	5.2	3	0	1000+	3	0
Orphan type IV (Weeks)	4.5	0	3	0	0	0
Coxsackie type A 9	4.5	0	0	0	1000+	0
Poliomyelitis type I (W. S.)	6.0	0	0	0	0	1000+

* Negative logarithm of titer per ml

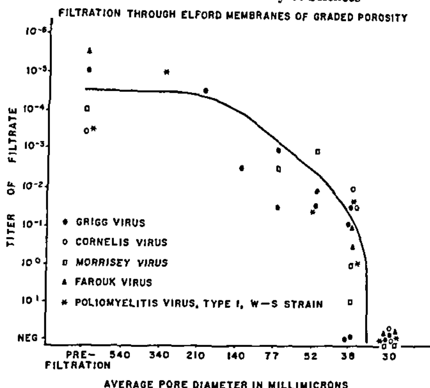


FIGURE 11 Comparative ultrafiltration data on three orphan viruses (Farouk strain, type 1 Cornelis strain, type 2, and Morrisey strain, type 3), a Coxsackie virus (Grigg strain, type A 9), and a poliovirus (W S strain, type 1) All the viruses were grown in monkey tissue culture for these experiments The filtration end point *lies between 30 and 38 mμ*

tration through gradacol membranes (FIGURE 11) In addition, Macrae, during his stay in New Haven, compared them in this set of experiments with a type I poliovirus and with a type A-9 Coxsackie virus grown in tissue culture under the same conditions All five viruses behaved alike, yielding values of 11 to 17 mμ for their diameters On the basis of comparative data on ultrafiltration, sedimentation and electron microscopy of poliomyelitis and of Coxsackie viruses,[39-41] the true diameter of types 1, 2, and 3 orphan viruses is probably about 30 mμ

It is noteworthy that the viruses which frequent the human alimentary tract —the poliomyelitis, the Coxsackie, and the orphan viruses—have much in common their size, their seasonal occurrence, their resistance to ether, and their *in vitro* cytopathogenicity for primate cells One wonders whether nature is about to yield another secret At least, the plot thickens!

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* These references are not meant to be a complete bibliography on the cultivation of poliovirus in tissue culture but merely to include certain recent papers in which methodology has been emphasized More extensive bibliographies of the early papers in this field have been published,[1, 2] and a detailed review of tissue culture methods and nutrient media used in poliomyelitis laboratories is in press[3]

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Discussion of the Paper

DOCTOR THOMAS H WELLER (*Harvard School of Public Health, Boston, Mass*): *Doctor Melnick has presented an admirable review of the status of* [illegible] procedures as applied to certain aspects of poliomyelitis research

[illegible]

sinized kidney suspensions prepared in India, and of the ingenious plastic [illegible] introduced by McLean are worthy of particular note. The latter innovation would have especial appeal to those of our technical staff engaged in the vaccine evaluation program, for each session of "tube-stoppering" produces its quota of blistered fingers

It has been emphasized that the choice of methods in different laboratories has been influenced by the availability of tissues or of other materials. There has been a natural tendency for a group of workers to advocate a particular technique, along with an implied endorsement suggesting its general applica-

bility. This monograph should serve to emphasize the need for methodological selection in terms of specific objectives. For example, the simplest of culture systems is desirable if the problem at hand entails the mass examination of sera [illegible]

maintain such cultures under optimum conditions for periods of three weeks or [illegible]

example, with Peebles, we have isolated a virus from man that destroys fibroblasts of monkey origin in a matter of hours, yet produces similar changes in human fibroblasts only after several days Again, in comparative titrations of Doctor Salk's three vaccine seed viruses (Saukett, Mahoney, MEF1), performed with Cheatham, we observed that cultures of human-kidney cells, as compared to monkey-kidney cells, were more rapid and slightly more sensitive indicators of viral activity in each instance

[illegible]

may be derived from components of the culture system *per se* On occasion, agents are manifest by their overt cytopathogenicity, as in the instance of those first observed in monkey-kidney cultures by Rustigian, or in the case of "B" virus Yet it is reasonable to suspect that other agents occur in a latent and commensal state One obvious explanation for the development of *in vitro* resistance in a cell line would envision the presence of a latent, interfering virus This is only one of a number of important lines of investigation deserving of further attention As Doctor Melnick has stressed, the technology of tissue culture as applied to the study of viruses is in a phase of rapid evolution.

A recent report by Salk, Youngner, and Ward[19] described a tissue culture test using the color of phenol red as indicator for poliomyelitis virus and antibody activity. The observation of inhibition of acid formation in infected cultures of tissue fragments had been noted in earlier studies with Western equine encephalomyelitis virus reported by Huang,[21] and with poliomyelitis viruses by Enders, Weller, and Robbins.[9] Several attempts to make use of inhibition of tissue metabolism as an indicator in roller-tube cultures were unsuccessful for reasons which have been described elsewhere.[19] The color test for poliomyelitis virus and antibody[19] utilizes standardized suspensions of monkey kidney tissue *prepared by an adaptation*[22] *of the trypsinization technique of Dulbecco and* Vogt.[23] A suitable quantity of trypsin-dispersed monkey kidney cell suspension is added to virus or virus-serum mixtures, before incubation. After six or seven days of incubation, the color of the phenol red in the medium serves as

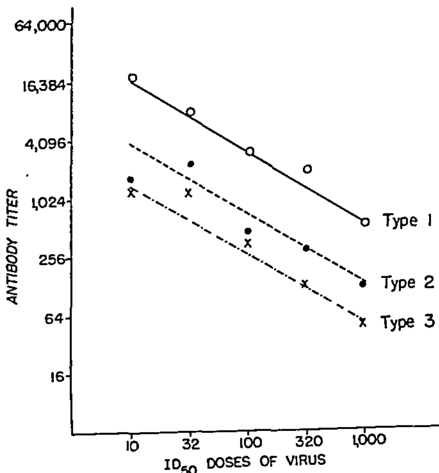


FIGURE 1. Influence of amount of antigen used on neutralization titers of poliomyelitis antisera. Roller tube cultures of monkey kidney fragments on glass (5 to 15 cultures per point).

TABLE 1

RESULTS OF REPLICATE TITRATIONS FOR TYPE 1 POLIOMYELITIS ANTIBODY BY USING THE COLOR TEST*

Test no	Dilution of type 1 antiserum† + 100 $TCID_{50}$ of type 1 virus‡				
	1,024	2,048	4,096	8,192	16,000
1	0/8	0/8	2/8	5/8	8/8
2	0/8	0/8	2/8	8/8	8/8
3	0/4	1/4	1/4	3/4	4/4
4	0/4	0/4	1/4	4/4	4/4
5	0/4	0/4	1/4	1/4	3/4
6	0/4	0/4	3/4	3/4	4/4
7	0/4	1/4	2/4	4/4	4/4
8	0/4	0/4	1/4	3/4	4/4
9	0/4	0/4	0/4	1/4	3/4
10	0/4	0/4	0/4	1/4	4/4
Cumulative score	0/48	2/48	13/48	33/48	46/48

* From Salk, Youngner & Ward.[19]
† Brunhilde No. A 453.
‡ Mahoney No. 25.

the indicator of virus or antibody activity. The presence of active virus results in cell death before there is enough growth and metabolism to cause the acid shift in the indicator color from red to yellow, which is observed in actively growing cultures. Death of the cells, by virus action, leaves the medium red. These procedures eliminate the necessity for preparation of cultures in advance, as is required for tests employing the microscopic indicator for virus or antibody activity in tube cultures. The use of indicator color as the basis for observing and recording end points has obvious advantages over microscopic readings of individual tubes.

The data in TABLE 1, taken from the report by Salk, Youngner, and Ward,[19] shows the results of a series of tests of type 1 antiserum made on different days, using different batches of trypsin-dispersed monkey kidney cells. It can be seen that, in 10 tests, the 1:2,048 dilution of serum gave a consistently protective effect, while the 1:16,000 dilution rarely protected. The 1:4,096 dilution did not protect in 13 of 48 tubes, and the 1:8,192 dilution failed in 33 of 48 tubes. Similar data have been obtained with types 2 and 3 antiserum titrations. The degree of variation inherent in the method for titrating serum antibody, using the color change indicator, is essentially the same as that encountered with other *in vitro* techniques such as complement-fixation and hemagglutination-inhibition tests, and has permitted the use of a single tube for each twofold dilution of serum when comparing acute and convalescent, prevaccination and postvaccination sera, or when carrying out antibody surveys in large populations.

The sealing of tubes in the color test by the insertion of a rubber stopper into each Wassermann tube has been replaced by a heavy mineral oil which is layered directly on the culture fluid in each tube to achieve the desired exclusion of air. Some of the interesting phenomena observed using such anaerobic tissue cultures have been described by Salk.[24]

Recent work by Robertson, Brunner, and Syverton[25] and by *Lipton an* Steigman[26] has shown that the tissue culture color test also can be *performe* using HeLa Cell suspensions. We have found that a *mineral-oil seal* can b used with *HeLa cell* cultures as well as with trypsin-dispersed monkey kidne cells. A somewhat accelerated fall in pH is observed under these anaerobi conditions

Studies have been made on the keeping qualities of monkey kidney cell suspensions used in the color test, and these indicate that the half life of the metabolic activity of cell suspensions is approximately five days when stored at 4° C under the conditions *employed* FIGURE 2 *shows* the details of a representative survival experiment A 1:50 dilution of cell suspension, stored at 4° C. was diluted serially in twofold steps, and the ability of *these dilutions to* produce an acid change in indicator color in seven days was tested. This procedure was repeated at daily intervals and, as can be seen from FIGURE 2, the *metabolic activity of the 1:100* and *1:200* dilutions was maintained for a period *of five days* The demonstrated stability of metabolic activity of kidney cell suspensions on storage suggests several possible practical applications First, it makes feasible the storage and utilization of cell suspensions for color tests *over a period of at least five days* The preparation of roller-tube cultures from cell suspensions stored for three days at 4° C. has been reported previ-

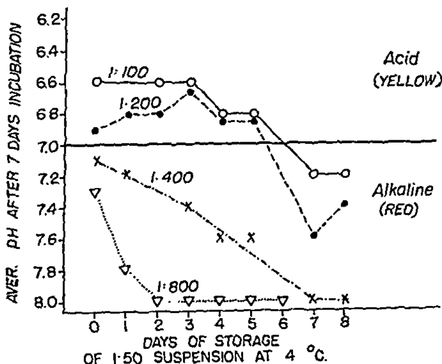


FIGURE 2 Influence of storage of 1:50 suspension of trypsin-dispersed monkey kidney cells (batch C-214) on stability of metabolic activity as measured by a tissue culture color test

TABLE 2

SUMMARY OF ANTIBODY TITERS FOR THREE PROTOTYPE HYPERIMMUNE MONKEY SERA WHEN TESTED BY DIFFERENT TECHNIQUES

Hyperimmune monkey serum	Virus* strain	Kidney				Testis fragments, plasma clots	Mice, I C
		Trypsinized cells		Fragments			
		Color test	Roller tubes	On glass	Under cellophane		
Type 1 Anti Brunhilde (A-453)	Mahoney	5,850† (3 7)‡	2,040 (3 3)	2,100 (3 3)	1,740 (3 2)	1,460 (3 1)	—
Type 2 Anti Lansing (A-300)	MEF 1	1,330 (3 1)	520 (2 7)	560 (2 7)	410 (2 6)	570 (2 7)	760 (2 8)
Type 3 Anti Leon (C-1108)	Saukett	—	—	390 (2 6)	—	340 (2 5)	—

* Approximately 100 ID_{50} doses of virus for the particular system involved were employed Viruses were used in the form of tissue culture pools

† Reciprocal of serum dilution producing a 50 per cent end point

‡ Log_{10} of serum dilution producing a 50 per cent end point

ously.[27] More recent information indicates that this period of viability is at least one week. Second, for purposes of economy and efficiency, trypsinized

with several antisera that have been tested by a variety of techniques in tissue cultures The type 2 serum also has been tested in mice The results show certain differences in antibody titers for each antiserum when tested in the different tissue culture systems In all cases, approximately 100 50 per cent infectious doses of virus for the particular system involved were employed. The finding that antibody titers obtained with the color test are approximately three times higher than those found with the cytopathogenic test in the various roller-tube systems, and possible explanations for this difference in sensitivity have been discussed previously[19] It has been pointed out that, in the color test, traces of antibody would be noted by their ability to delay virus activity. This would be evidenced by a pH drop before sufficient virus destruction to inhibit metabolic activity completely took place In the roller-tube system, any degree of severity of cellular degeneration would be considered a failure of the serum to protect the culture, and traces of antibody noted in the color test would not be recorded as such in the microscopic cytopathogenic test

An attempt has been made in this paper to bring together information on the methods for demonstration of neutralizing antibodies for poliomyelitis viruses Technical procedures have been so simplified that quantitative determinations of poliomyelitis antibody can be carried out in large numbers with relative ease

References

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Recent work by Robertson, Brunner, and Syverton[25] and by Lipton and Steigman[26] has shown that the tissue culture color test also can be performed using HeLa Cell suspensions. We have found that a mineral-oil seal can be used with HeLa cell cultures as well as with trypsin-dispersed monkey kidney cells. A somewhat accelerated fall in pH is observed under these anaerobic conditions.

Studies have been made on the keeping qualities of monkey kidney cell suspensions used in the color test, and these indicate that the half life of the metabolic activity of cell suspensions is approximately five days when stored at 4° C. under the conditions employed. FIGURE 2 shows the details of a representative survival experiment. A 1:50 dilution of cell suspension, stored at 4° C. was diluted serially in twofold steps, and the ability of these dilutions to produce an acid change in indicator color in seven days was tested. This procedure was repeated at daily intervals and, as can be seen from FIGURE 2, the metabolic activity of the 1:100 and 1:200 dilutions was maintained for a period of five days. The demonstrated stability of metabolic activity of kidney cell suspensions on storage suggests several possible practical applications. First, it makes feasible the storage and utilization of cell suspensions for color tests over a period of at least five days. The preparation of roller-tube cultures from cell suspensions stored for three days at 4° C. has been reported previ-

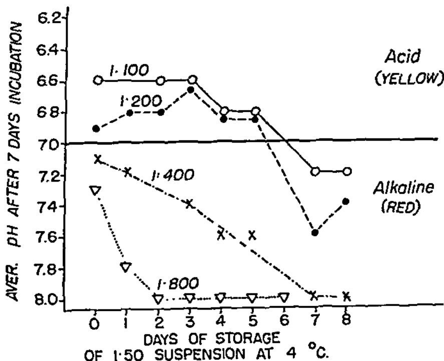


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Type 3 Anti Leon (C 1108)	Saukett	—	—	390 (2 6)	—	340 (2 5)	—

* Approximately 100 ID_{50} doses of virus for the particular system involved were employed Viruses were used in the form of tissue culture pools

† Reciprocal of serum dilution producing a 50 per cent end point

‡ Log₁₀ of serum dilution producing a 50 per cent end point

ously[27] More recent information indicates that this period of viability is at least one week Second, for purposes of economy and efficiency, trypsinized cell suspensions could be prepared in a central laboratory and, perhaps, even in facilities situated close to the source of supply of monkeys, and then shipped under refrigeration by air for use in widely separated laboratories

TABLE 2 has been prepared to show a comparison of antibody titers obtained with several antisera that have been tested by a variety of techniques in tissue cultures The type 2 serum also has been tested in mice The results show certain differences in antibody titers for each antiserum when tested in the different tissue culture systems In all cases, approximately 100 50 per cent infectious doses of virus for the particular system involved were employed The finding that antibody titers obtained with the color test are approximately three times higher than those found with the cytopathogenic test in the various roller-tube systems, and possible explanations for this difference in sensitivity have been discussed previously.[19] It has been pointed out that, in the color test, traces of antibody would be noted by their ability to delay virus activity This would be evidenced by a pH drop before sufficient virus destruction to inhibit metabolic activity completely took place In the roller-tube system, any degree of severity of cellular degeneration would be considered a failure of the serum to protect the culture, and traces of antibody noted in the color test would not be recorded as such in the microscopic cytopathogenic test

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POLIOMYELITIS COMPLEMENT-FIXING ANTIBODIES AND THEIR DEMONSTRATION

By Francis L. Black

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A complement-fixation reaction in the poliomyelitis system was demonstrated by Casals, Olitsky, and Anslow[1,2] in 1950 by means of an antigen from a suckling mouse-adapted strain of type II virus. These workers showed that sera from many individuals of the normal population of this country reacted with [illegible] a more satisfactory source of antigen, and studied the response of patients to the three types of virus.

No special techniques are required to demonstrate the complement-fixation reaction. Most workers in the field use the Fulton and Dumbell plate technique[4] because of the convenience of this method and its economy in materials. It is possible to use any one type of antigen alone or a combination of the three types in testing sera. The latter procedure is useful for screening predominantly normal sera. A typical test plate is shown in FIGURE 1. On this plate, one serum sample has been run against antigens of the three types at each of several levels of complement. A serum control is included as a check for anticomplementary factors in the serum. With each series, controls would also be run to test each of the other reagents for anticomplementary activity. The cells that remain unhemolyzed are agglutinated to form a button in the center of the drop. Complement-fixation is determined by the size of the button. In this example, there is fixation in the presence of the type I and, to a lesser extent, of the type III antigens. The several concentrations of complement in each test give checks on the serum titers, allow one to estimate the effect of anticomplementary sera, and also give a value for the strength of the reaction based on the amount of complement fixed, a value which complements that of the limiting dilution titer in describing the characteristics of a serum.[5]

Tissue culture fluids from monolayer cultures of HeLa or monkey kidney cells [illegible]

The relationship between the C-F activity and infective particles has been discussed by the previous speakers. The antigen is stable for several months at 5° C. Incubation of virus at 56° C. for 30 minutes will destroy the infectivity without significantly reducing or broadening the antigenicity. Use of heated antigen, instead of fresh, reduces the danger of laboratory infection. Formalinization under conditions that destroy the infectivity of the virus may also leave the antigenicity intact.

FIGURE 1 A complement fixation test plate showing the titration of a serum against the three poliomyelitis antigens, and a control, one in each quadrant. One unit of complement was insufficient to lyse a significant proportion of the cells while two units lysed most of them, except where complement had been fixed

Standard monotypic sera may be obtained by the injection of monkeys or chimpanzees with a single type of virus

The complement-fixing activity of normal human populations varies from one population group to another Where primitive sanitation conditions exist, the C-F antibodies are largely confined to children Of children three to four years old, 60 to 70 per cent may have antibodies against one or more types, but only a few per cent of the adults in these areas have measurable titers In the United States, on the other hand, the peak frequency of C-F

antibodies is not reached until later in childhood, and the number of positive reactions remains high in the adult population.

The complement fixation test is the third of the laboratory tests, useful in the identification of poliomyelitis infections, discussed in this monograph. A complement-fixation test has certain inherent advantages over both virus isolation and neutralization tests. A single test may be carried out in 24 hours, less labor is involved than in either of the other two tests, and the test may be carried out under less stringent safety precautions, since infectious virus is not needed. The reactions that have been found in the poliomyelitis system, however, are often complex and, as the test is used at present, less than 50 per cent of cases show an increasing titer against only one type. More than 90 per cent of those from whom virus can be isolated possess a high antibody titer against one or more types.

When paired sera from poliomyelitis cases are tested, a number are found to possess C-F antibodies against one type only. Some examples of these monotypic reactions are given in TABLE 1. During the first week after onset, the titers are usually low or negative, and it is thus possible to obtain acute phase values that provide a marked contrast to the convalescent. After the first week, the titers rise and remain high for several months. Most of these cases also give monotypic neutralizing reactions, but some give transitory heterotypic neutralizing reactions, and some have heterotypic neutralizing antibodies in both acute and convalescent sera. The homotypic neutralizing antibodies usually reach a high titer before the complement fixing. In the cases the author has studied, where virus has been isolated from individuals who gave a monotypic C-F reaction, the virus has always been of the same type as the C-F antibody. Hence, in a case such as the third example, where no virus was isolated, the increasing monotypic C-F titer is presumptive evidence of the nature of the infection.

Many poliomyelitis patients possess C-F antibodies against two or three types of virus (TABLE 2). In these cases, the antibody usually develops rapidly, sometimes being present in high titer on the day of onset (example 4). The changes in titer between paired sera may occur in almost any combination

TABLE 1
EXAMPLES OF MONOTYPIC C-F RESPONSES IN POLIOMYELITIS PATIENTS

	Virus isolated	Days after onset	C-F titer			Neutralizing titer		
			I	II	III	I	II	III
1	I	4	0	0	0	100+	0	0
		21	32	0	0	100+	0	0
		41	32	0	0	100+	10	0
2	II	6	0	0	0	32	100+	0
		28	0	8	4	0	100+	0
		49	0	32	0	0	100+	0
3	Neg	3	0	0	0	100+	0	0
		21	4	0	0	100+	0	0
		50	16	0	0	100+	0	0

TABLE 2
EXAMPLES OF HETEROTYPIC C-F RESPONSES IN POLIOMYELITIS PATIENTS

	Virus isolated	Days after onset	C-F titer			Neutralizing titer		
			I	II	III	I	II	III
1.	I	7	0	0	16	160+	20	0
		49	32	0	4	160+	40	0
		70	32	0	0	160+	160+	0
2	I	7	±	0	±			
		15	32	0	32			
3	III	4	32	0	32			
		130	8	0	32			
4	II	1	16	8	8	100+	100+	0
		24	8	8	4	100+	100+	0

the homotypic titer may rise as the heterotypic falls (example 1); the titer against more than one type may rise simultaneously (example 2), or one or another type may be approximately the same in acute and convalescent specimens (examples 3 and 4) Frequently, the type of the antibody showing the greatest increase in titer agrees with the type of virus isolated from the stool, but there are exceptions, and little reliance can be put on such a result for identification of the type of the infecting virus Most individuals who show heterotypic C-F responses possess neutralizing antibodies of more than one type, but not necessarily of the same types as their C-F antibodies C-F antibodies may occur when no neutralizing antibodies of that type are demonstrable. The work that has been done on these heterotypic responses to the present time does not allow a definition of what part infections with pathogens other than poliomyelitis may play in their development. If it should prove that few other infections give rise to a poliomyelitis C-F response, the usefulness of the C-F test would be greatly enhanced, as any high titer might be taken as an indication of recent contact with poliomyelitis or a small group of other agents In order to use the test in this manner, one would first need to eliminate, from the antigen, impurities of monkey origin that might give a false reaction with some sera

[illegible] group of cases in which the C-F antibodies never [illegible] ed The [illegible] It may be that many of these negative results are actually due to a masking of complement fixation by other reactions, and not to a lack of antibody production.

The fraction of a group of individuals giving monotypic or polytypic reactions depends, among other things, on the ratio of the reagents used in the test system Reduction of the antigen concentration to two units reduces the heterotypic reactions but, at the same time, eliminates some of the weak monotypic reactions and thus increases slightly the number in which no antibody is detected

Different population groups give different proportions of monotypic reactions On one group of 40 persons, from whom virus was isolated, 80 per cent

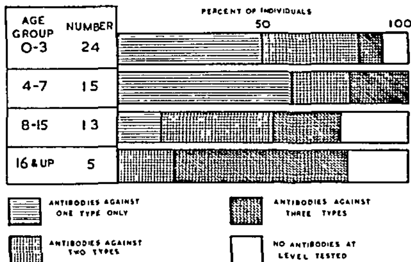


FIGURE 2 Age distribution of C-F reactions in poliomyelitis patients

gave monotypic increases in C-F antibodies[6] In another group, from a center less than 150 miles away from the first, only 38 per cent gave monotypic reactions when tested by the same technique.

Young children give a higher percentage of monotypic reactions than do the older age groups The reactions of a number of individuals are broken down into the respective age groups in FIGURE 2 The proportions in each reaction category in this figure hold only for this particular group of 57 persons as tested by one method, and cannot be considered necessarily representative of the pop-

C-F response may be largely an anamnestic recall of heterotypic antibodies There is another piece of evidence that supports this hypothesis A group of 30 persons who received a single injection of formalin inactivated type I virus were tested for neutralizing and for C-F antibodies before and after inoculation No C-F antibodies were found in this particular group prior to injection The C-F response was confined almost entirely to those persons and those types where there was pre-existing neutralizing antibody A breakdown of this group is given in FIGURE 3 into those with positive or negative prevaccinal neutralization reactions and within these categories according to C-F response

There is still much to be learned about the optimum conditions for carrying out the test, about the relative importance of past experience and strain and

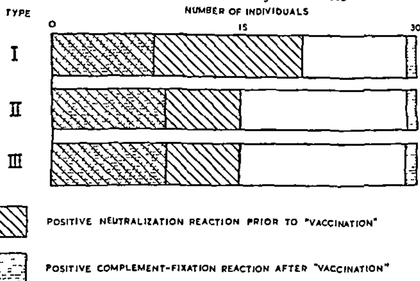


FIGURE 3 Pre-existing neutralizing antibodies and C F response to formalinized type 1 virus

individual differences in determining the nature of the response, about the role of nonpoliomyelitic viruses in stimulating an increase in poliomyelitis C-F titer, and about the antigenic composition of infectious and inactivated virus preparations As it stands at present, the C-F reaction allows identification of a significant proportion of poliomyelitis infections, but less than either the virus isolation or neutralization tests Because of its other advantages, it is of practical use, particularly when dealing with young children, both as an independent test and as confirmation of other tests

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3 S[illegible]
4. F[illegible]
5 B[illegible]
6 MELNICK, J [illegible]
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Discussion of the Paper

DOCTOR MANFRED M MAYER (*School of Hygiene and Public Health of the Johns Hopkins University, Baltimore, Md*). From the papers that have just been presented, it is clear that rapid advances have been made in the development of techniques for studying the serology of poliomyelitis It is my pur-

pose to discuss briefly the interpretations, some of the limitations, and certain technical aspects of these tests

First, it should be recognized that the technics for neutralization and complement-fixation tests, as commonly used, and with the exception of the studies presented by Doctor Dulbecco, are still rather crude and require continual refinement There is need for studying in detail the many factors which influence the outcome of these tests For example, in the neutralization test, the concentrations of cells and virus constitute important factors which must be carefully controlled The question of noninfective but antigenically active virus requires investigation In the complement-fixation reaction, the concentrations of red cells and of complement, as well as that of antigen, play an important role. There is need for further studies on the effect of temperature of incubation, the concentration of Ca^{++} and, perhaps most important of all, the choice of the strain of virus from which the antigen is prepared In addition, there are indications that the "so-called" indirect complement-fixation test may prove to be of considerable value Altogether, it seems to me that we know far too little as yet about the complement-fixation reaction in polio, and its use on a practical level is premature

The impression may have been created by opinions expressed by some of the contributors to this monograph that the complement-fixation reaction presents technical advantages and may therefore be preferred to the neutralization test, at least under certain circumstances I think this is a misleading viewpoint, since we have found, in confirmation of the findings of other workers, that the results of complement-fixation tests and of neutralization tests are not necessarily interchangeable, and not always comparable As in a number of other virus infections, it would seem that the polio antibodies detected by neutralization are not necessarily the same as those observed in complement-fixation

For example, in a number of polio cases under study in our laboratory, we have observed an increase in complement-fixing antibody for a type of polio virus other than that causing the infection Furthermore, changes in complement fixation titer frequently do not run parallel to those in neutralization tests The source of discrepancies of this nature has been somewhat of a puzzle to investigators in this field and deserves discussion The difficulty of interpretation arises from the unitarian hypothesis, *i e*, the concept that one antibody can elicit a variety of immunological reactions, including precipitation, neutralization, complement fixation, agglutination, anaphylaxis, and others, depending on the choice of experimental conditions and the choice of the form of the antigen This idea has become well established on a sound experimental basis, and therefore one must ask why it should fail to apply to many viral antigens in general and in poliomyelitis in particular

I should like to advance two reasons for this First, it has been shown that the complement-fixing efficiency of antibody, *i e*, the capacity of a given weight of antibody to fix complement, can vary, depending on the manner and intensity of the antigenic stimulus I think this effect might account, at least in part, for the observation common to many virus systems that complement-

fixing antibodies disappear from the circulation more rapidly after infection than neutralizing antibody

This interpretation, however, fails to account for the observation relating to discrepancies of specificity between complement-fixation and neutralization In order to approach this problem I should like to propose consideration of a second factor. It has been shown recently by Dulbecco that the neutralization of polio virus by antibody is a one-hit reaction, *i e.*, a virus particle can be inhibited by combination with a single molecule of antibody. From geometric considerations and by analogy with numerous other antigen-antibody systems, it can be estimated that one particle of virus can combine with perhaps 50, 100, or even 200 molecules of antibody. It is difficult to see how a single molecule can block the infectiveness of the virus unless the assumption is made that this inhibiting molecule of antibody enters into combination with a key-site or a specific active group on the virus particle which is intimately concerned with and is essential for its capacity to infect a susceptible cell. If the additional, rather likely assumption is made that the surface of the virus particle possesses a variety of antigenic determinant groups, one is led to the probable supposition that the key-site for infection may possess a distinct antigenic pattern and that, therefore, the neutralizing antibody comprises those antibody molecules in a serum which are capable of combining with this antigenic pattern. This would mean that, in a neutralization test, the detection of *antibody does not involve all of the antibody molecules corresponding to the* various determinant groups of the virus particle, but only those which can react specifically with the key site or sites In other words, in a neutralization test we are presumably detecting a special kind of antibody. By contrast, it appears likely that in complement-fixation reactions all of the antibody or, at [illegible]

in the complement-fixation reaction.

This line of reasoning receives support from observations in the neutralization of bacteriophage by antibody. Its implications with respect to polio become quite clear if it is recalled that the establishment of the three recognized types of [illegible] *fectivity* [illegible] postulated [illegible] viruses, which differ markedly in the specificity of the key-site antigen(s), may have in common many of the antigenic determinant features associated with other parts of the surface of the virus particle Therefore, in a complement-fixation reaction in which all, or at least most, of the determinant groups and their corresponding antibodies participate, it is quite possible that extensive cross reaction among the types would be encountered It is possible that other factors play a role but, for the purpose of this limited discussion, I should like to confine my remarks to the two factors cited.

Recent complement-fixation experiments performed in this laboratory with highly purified type 2 and type 3 poliomyelitis viruses and human poliomyelitis

sera* have yielded results that are directly comparable in terms of specificity with those obtained by neutralization tests, except for much lower titers in the [illegible]

[illegible]

used for propagation of the virus

* Purified virus kindly supplied by Doctor Carlton E. Schwerdt.

BIOLOGICAL PROPERTIES OF POLIOMYELITIS VIRUSES AS STUDIED BY THE PLAQUE TECHNIQUE*

By R Dulbecco and Marguerite Vogt
Kerckhoff Laboratories of Biology, California Institute of Technology, Pasadena, Calif

The plaque technique for animal viruses was developed for the purpose of providing a method suitable for a quantitative analysis of the interrelations between animal viruses and their host cells So far, this method has been applied to a number of problems in the field of poliomyelitis viruses, such as the intracellular growth of poliomyelitis virus, its release from infected cells, its genetic properties, and its inactivation by antibody In the present communication, we shall discuss some results obtained in the study of the growth and genetic properties of poliomyelitis virus

Before describing the experimental results, let us discuss a point of theoretical importance, namely the characterization of the virus particle.

We detect a virus particle by its effect on a monocell layer, namely that of giving rise to a localized necrosis of the cell layer, a so-called plaque. Each plaque contains a viral population stemming from a single virus particle This is proved by the following two findings According to the first finding, the number of plaques produced by a virus sample is proportional to the amount of the sample deposited on the cell layer in dilution experiments (R. Dulbecco and M Vogt, 1954*a*) This result proves that either a single virus particle or a clump of particles, indivisible upon dilution, is sufficient to produce a plaque The second finding is the linearity of the virus inactivation curves obtained by ultraviolet light (FIGURE 1) In these curves, the survival of the plaque-forming ability of the virus is measured as a function of the dose of ultraviolet light to which the virus was exposed If the virus particles were aggregated in clumps, the plaque-forming ability of the clump would be destroyed only after destroying the plaque-forming ability of *all* the individual particles present in the clump It can be demonstrated, and is intuitively obvious that, under these circumstances, the resulting inactivation curves would have a lag at low doses Such a lag is not found experimentally.

The results just mentioned clearly define the concept of the plaque-forming particle as a physical and biological unit Since the plaque-forming titer of a virus preparation is of the same order of magnitude as the infectious titer determined by other methods, the plaque-forming particle can be identified with the infectious particle Furthermore, since the operational definition of a virus particle rests on its infectivity, this infectious particle is *the virus particle*

It has now to be discussed whether the counting of plaques gives us an accurate estimate of all the virus particles present in a given preparation This discussion is the more required since purified preparations of poliomyelitis virus have been shown by electron microscopy (C E Schwerdt *et al*, 1955) to contain 100 to 1000 times more particles than the corresponding estimate from plaque counts We shall discuss the problem for a system constituted by

* This work was aided by a grant from the National Foundation for Infantile Paralysis, Inc New York, N Y.

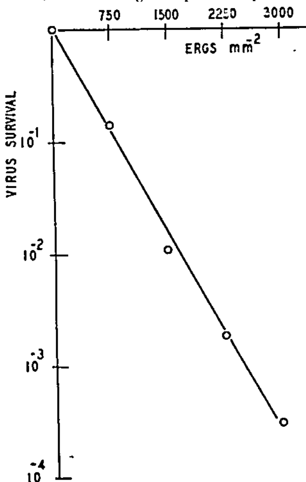


FIGURE 1. Ultraviolet inactivation curve of poliomyelitis virus type 1, Brunhilde strain. Two milliliters of a virus suspension, diluted 1:250 in an ultraviolet transparent medium, were irradiated in a 10 cm. watch glass. 0.1 ml. samples were withdrawn at regular intervals. The source for the UV light was a "germicidal" lamp.

type 1 Brunhilde virus and monkey kidney cells in the usual monolayer conditions.

A first possibility to be considered is that all particles in these purified virus preparations are infectious but remain mostly undetected, due to a low probability of becoming attached onto the cell layer. This possibility may be excluded on the following grounds: during the 30 minutes of contact between the virus and the cell layer approximately one third of the infectious particles become attached to the cell layer, since they disappear from the virus preparation and appear as plaques. If, on the other hand, only one out of a thou-

sand particles became attached, no disappearance of plaque forming particles from the virus preparation would be detected. Disregarding the fact that further virus particles may still become attached later when the agar overlay has been added, an underestimate of the infectious particles present in the sample due to incomplete adsorption will not surpass a factor of three

A second possibility accounting for the excess particles in the purified preparations would be that most of the adsorbed particles become attached to nonsusceptible cells This possibility can also be excluded Experiments with infected cell suspensions obtained from monolayer cultures show that at least half of the cells of a layer can be infected independently and are able to release progeny virus. Thus, the number of plaques is not limited by the number of susceptible cells as, for instance, in plant virus assays. Considering both possibilities together, the number of undetected plaque-forming particles in a given virus sample can at most be six times the number of plaques actually observed.

[illegible]

two following reasons

The first reason is that these particles would also lack the ability of interfering with the growth of infectious particles added later, as may be deduced from the following experiment If a cell layer is infected with a dose of cytopathogenic virus able to infect 10 per cent of the cells, all the cells of the layer should be infected by the hypothetical noncytopathogenic virus particles. Nevertheless, it can be observed that the cells that were not infected by particles of the cytopathogenic virus at the beginning of the experiment are infected later by cytopathogenic virus particles released in the first cycle of infection These cells show the same cytopathogenic changes as the cells infected first and release cytopathogenic virus, as indicated by the final titer reached

The second reason that makes it unlikely that the particles in question are virus particles is the fact that cells infected with poliomyelitis virus would yield from 10^5 to 10^6 virus particles, a number surpassing by far the number of 10^{3-4} virus particles characteristic of the yields of cells infected by all other known cytopathogenic viruses

It seems likely, therefore, that the nonplaque-forming particles lack the main biological characteristics of virus particles The relation between the physical particles and the infectious virus particles is still an open problem of highest interest

We wish to comment briefly on some chemical properties of the virus par-

[illegible]

ticles in the purified preparations appear to be noninfectious and of unknown relationship to the infectious virus particles, as already discussed, the results of the chemical analysis may not reflect the true composition of the infectious

virus particles. It was thought, therefore, that some information on this point could be obtained indirectly by studying the effect on the virus infectivity of substances able to act selectively upon RNA or DNA

On the one hand, the action of RNAase and DNAase was tested. Both enzymes were found to have no effect on the infectivity of Brunhilde virus at 37° C The action of RNAase and DNAase was also studied at a temperature of 43° C, under the assumption that a reversible denaturation of the virus protein at this temperature might render the virus nucleic acid accessible to the enzymes. Under these same conditions, however, no significant virus inactivation was obtained.

On the other hand, the action of N-mustard gas on Brunhilde virus was determined. It is known that DNA viruses (such as phages) and the DNA-containing transforming principles are heavily damaged by nitrogen mustard whereas RNA viruses (such as tabac mosaic virus) are very little affected (R. M. Herriott, 1948). It was found that Brunhilde virus is surprisingly resistant to nitrogen mustard, much more so than viruses previously found to be very resistant, such as Western equine encephalomyelitis virus (cf FIGURE 2) Although the meaning of this result is not yet fully understood, it may support the notion that DNA is no essential component of poliomyelitis virus

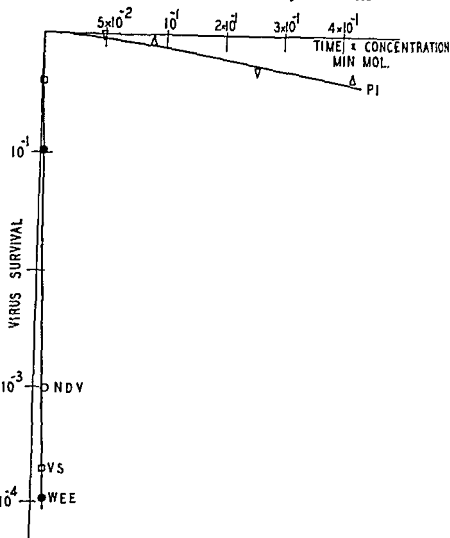
We now turn to the study of the growth properties of the poliomyelitis viruses One-step growth curves of the three types of poliomyelitis virus have been determined on suspensions of isolated monkey kidney cells. The conditions of a one-step growth experiment have been previously described (R Dulbecco and M Vogt, 1954b), they insure that no progeny virus is lost due to readsorption of released virus to the cells The medium was a mixture of monkey serum, chicken embryo extract, and Earle's saline

The growth curves of the three types shown in FIGURE 3 are very similar except for minor differences in the lengths of the latent period

Growth curves of type 1 virus were also determined for suspensions of

The study of the release of virus from individual cells in microdrops was carried out in collaboration with Doctors A and M Lwoff The results of this study will be the object of a separate communication (A Lwoff *et al*, 1955)

A first attempt was made to study the intracellular growth of poliomyelitis virus by following its resistance to UV irradiation during various phases of intracellular virus growth, a technique previously used in bacteriophage work (S E Luria and R Latarjet, 1947, S Benzer, 1952) So far, the sensitivity of the virus to UV irradiation was determined immediately after its adsorption to monkey kidney cells (time 0) and after one hour of intracellular virus growth. To obtain the UV-sensitivity of the virus at time 0, the virus was adsorbed to the cells in the presence of cyanide To reduce the screening effect of the cells, the virus infection and irradiation were done on monolayer cultures in which the cells are well spread and therefore very thin Under these conditions, the



screening by the cells was found to be negligible, since not more than 15 per cent of the incident light was found to be absorbed by monolayer cultures After the irradiation, the infected cells were brought into suspension by exposure to a sodium versenate solution (which yields a cell suspension containing practically only single cells) The number of cells capable of releasing virus

was determined by the plaque technique. The numbers observed were corrected for the loss of the ability of the *cells* to support virus growth at the corresponding UV doses The necessary correcting factor was obtained from an independent determination of the survival curve of the ability of the cells to support virus growth if irradiated *before* infection

As seen from FIGURE 4, the results of these experiments showed a very marked increase of the UV resistance of the virus immediately after adsorption followed by a further, although less pronounced, increase, during the first hour of intracellular virus growth These changes in the UV resistance show that the virus is deeply modified in its physical properties immediately after adsorption. The virus particle may be broken down and only a fraction of it may proceed to further intracellular growth, the structure of the virus particle may have changed greatly, or virus components may have interacted with cell

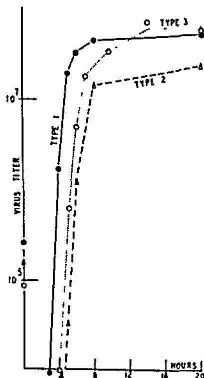
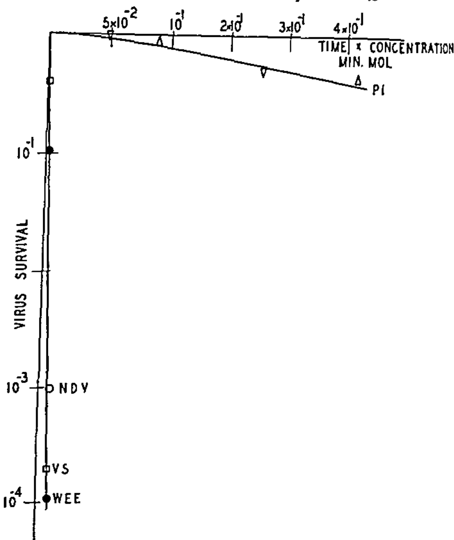


FIGURE [illegible]. One step growth curves of poliomyelitis virus type 1 (strain Brush...) type 2 (strain Y SK), and type 3 [illegible]



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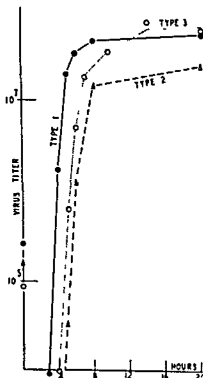


FIGURE 1. One step growth curves of poliomyelitis virus type 1 (strain Brunhilde), type 2 (strain YSK) and type 3 [illegible]

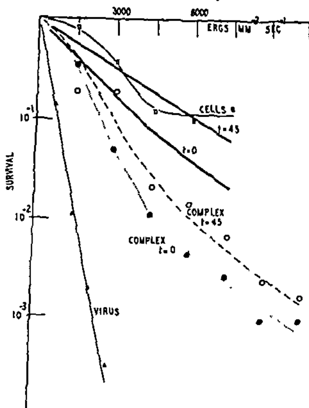


FIGURE 3
(Brunhilde strai
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and 45 minutes
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minutes, respect

components Forthcoming X-ray studies will cast additional light on this problem.

We now turn to another object of our study, that of the genetic properties of poliomyelitis viruses Since plaques originate from single virus particles, they provide a proper source for the genetic purification of virus strains (R. Dulbecco and M Vogt, 1954a) Of course, we cannot state that the virus population present in a plaque is genetically uniform, since new mutations may have occurred, or hypothetical multiple genetic factors have segregated during the development of the plaque

It appears *a priori* possible that variant virus strains may be identified by and isolated from plaques of special appearance Such special plaques do not occur in virus stocks at such a frequency that they can be isolated by inspecting a limited number of plaques Selective procedures, therefore, must be employed The method of rapid serial passages of large virus populations on cells of a certain type, a method previously used by other authors, has been adopted by us as a selective method

An 18th tissue-culture passage stock of type 1 virus, Brunhilde strain, was passed through serial rapid passages on monolayer cynomolgus kidney cells. At the 15th passage, a plating of the virus revealed several plaques of different type, characterized by their larger size and their sharper rim. One of these plaques was picked up and gave rise to a virus line which, after further purifi-

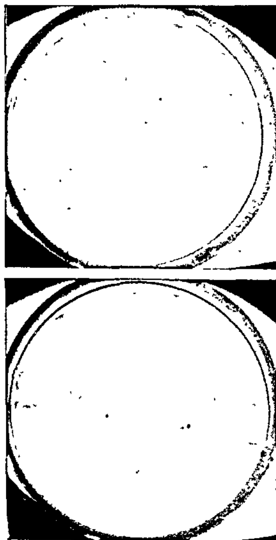


FIGURE 5. Plaque morphology of the r mutant and of the wild type: r mutant, upper; wild type, lower. The two virus lines were plated on parallel monolayer cultures of monkey kidney cells and incubated for four days. 60 mm. Petri dishes.

of the *r* mutant during the rapid serial passages on monolayer kidney cultures the growth characteristics of the *r*-mutant were determined and compared with those of the wild type. As seen in FIGURE 6, no significant difference in the length of the latent period nor in the slope of the exponential rise period between the mutant and the wild type were found. This holds both for the growth curves obtained in a medium containing monkey serum or a medium containing embryo extract only. The unfavorable effect of the absence of monkey serum was equally effective on the growth curves of the *r* mutant as on that of the wild type and expressed itself in a longer latent period and a slower rise during the exponential rise period Whereas the characteristics of the growth curves account for the different plaque development of either virus line in the two different media, they fail to explain the difference between *r* and wild type plaques in embryo extract medium

A selective factor, responsible for the enrichment of the *r*-mutant during the serial passages, therefore, could not be demonstrated This failure may be interpreted in two different ways:

(1) It is conceivable that the enrichment was brought about by selective factors other than the rate or extent of growth, or that the over-all selective advantage of the variant type over the wild type at each generation was very small, but may have produced a considerable enrichment over repeated passages, each involving several virus generations.

(2) The variant may have been isolated not be selection pressure, but by mutation pressure It will be necessary to await the results of many additional experiments in order to distinguish between these interpretations and to define the role of the host cells in the process by which the variant strain was obtained

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KINETICS OF THE RELEASE OF POLIOMYELITIS VIRUS FROM SINGLE CELLS*

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The experiments described in this report were initiated with the ultimate goal of studying the problem of the interrelation of viruses and cells at the level of individual cells. The technique used was that of DeFonbrune, which has been already applied to the study of the release of virus from lysogenic bacteria (A. Lwoff and A. Gutmann, 1950). In this technique, the cell to be studied is contained in a small drop of nutrient medium immersed in paraffin oil, which is permeable to gases

The following experimental procedure was employed A suspension of monkey kidney cells was prepared by dispersing the cells of a monolayer culture with trypsin or versenate. The cell suspension was then infected with poliomyelitis virus, type 1 (Brunhilde strain). After an adsorption time of 30 minutes, the remaining free virus was eliminated by centrifugation The infected cells were then suspended in a culture medium containing 10 per cent monkey serum and 20 per cent chicken embryo extract, at a final concentration of 10^5 cells per ml This time was taken as time 0. Individual infected cells were isolated in microdrops in the following way A clean coverslip was placed in a leucite box on the stage of a dissecting microscope and covered with paraffin oil The leucite box was flushed with a mixture of air and 5 per cent CO_2 The microscope was contained in a thermostatic box maintained at 37° C A capillary pipette containing the cell suspension was introduced through a lateral opening of the leucite box Under microscopic observation, a number of microdrops corresponding to a volume of about 4×10^{-6} ml of the cell suspension were deposited on the coverslip under the paraffin oil The drops adhered to the glass displacing the oil Care was taken that each microdrop contained only one cell

The infected cells attached to the glass, and a proportion of the cells spread within one to three hours Two different procedures were used to study the release of the virus In the first procedure, the coverslip was left permanently in the leucite chamber Samples were withdrawn at regular time intervals from the microdrops in the following way A large fraction of the liquid of the drop was sucked into a capillary pipette and substituted by fresh medium, and the operation was repeated a second time This procedure allowed recovery of 95 to 99 per cent of the fluid of the original drop The two samples were pooled into 0.2 ml of Earle's saline containing 20 per cent embryo extract and frozen The number of virus particles contained in the various samples was determined by the plaque technique

The second procedure for studying the release of the virus involved an additional step The coverslip carrying the microdrops was removed from the

* This work was aided in part by a grant from the National Foundation for Infantile Paralysis Inc New York N Y and in part by a grant from Abbott Laboratories Teterboro N J

leucite chamber and inverted over a DeFonbrune micromanipulator chamber which was then filled with oil. The chamber was placed on the stage of a phase-contrast microscope contained in a thermostatic box which also contained a DeFonbrune micromanipulator. The box was maintained at 37° C. and flushed continuously with a mixture of air and 5 per cent CO_2. In this second series of experiments, the virus released by the isolated cells was again determined by withdrawing samples from the microdrops at various time intervals The operation, however, was carried out by using a micropipette operated with the help of the micromanipulator. In addition, the morphological changes of the cell were followed and recorded photographically

The results of both series of experiments are shown in TABLE 1 and FIGURES 1 to 4.

TABLE 1 brings the virus release from two cells which was followed at regular intervals of half an hour As may be seen from the table, cell 1 released the majority of the virus particles between six and six and one-half hours after the

TABLE 1

VIRUS RELEASED FROM TWO ISOLATED MONKEY KIDNEY CELLS INFECTED WITH POLIOMYELITIS VIRUS, TYPE 1 (BRUNHILDE STRAIN)

Number of cell	Number of plaque forming particles released at various times after infection								
	Hours								
	4	4½	5	5½	6	6½	7	8	9
1	0	0	0	0	>120	50	4	0	21
2	0	0	0	0	0	0	>89	21	6

adsorption of the virus, cells 2 between seven and seven and one-half hours after adsorption (since the two samples taken at nine hours contained also the cell, the virus particles found in these samples may have been associated with the cell and been released when the latter was disrupted by freezing) The results show, thus, that the bulk of the virus was released by the cells within one hour

... growth curves ob-

to the average virus yield obtained from infected cell suspensions A certain degree of variability was found with respect to the latent period of the different cells To assess the full range of this individual variability, however, many more experiments will have to be done The latent period shown by the four cells is, in addition, longer than the latent period in a mass culture This may be due mainly to technical factors introduced by the manipulation of the cells

The main purpose of the second series of experiments was to correlate the release of the virus with certain morphological changes of the cells FIGURE 2

[illegible] of these morphological changes. The [illegible] release [illegible] as follows: One or two hours before the virus release begins, the cell contracts and withdraws its pseudopodes. Later on, a hyaline zone at the periphery of the cell appears and seems to precede immediately the virus release. The formation of a hyaline zone may not be pronounced if the cells are very contracted. The hyaline zone undergoes a pronounced vacuolization during the time of release. At the time the virus release ends, there is extensive cell disruption.

The correlation between the virus release and the morphological changes just described is further supported by FIGURES 3 and 4, which represent the changes shown by two other virus-releasing cells. In the case of FIGURE 4, most of the virus samples were taken at the time of the vacuolization of the hyaline zone. As may be seen, this time corresponded to the period of continuous virus release.

The morphological picture thus suggests that the release of the virus occurs through lysis of a part of the cell. This notion is supported by the finding, obtained in collaboration with Doctor Cooper, that monolayer cultures of P32 labeled monkey kidney cells infected with poliomyelitis virus of the same strain, release approximately half of their label simultaneously with the virus. It was not yet possible to study the morphological behavior of the nucleus in the infected cells due to the rounding up of the cells before the release of the virus.

The results show that it is relatively easy to study the virus yields from individual cells, and it will be of interest, therefore, in the future, to compare the

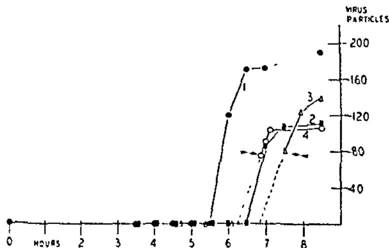
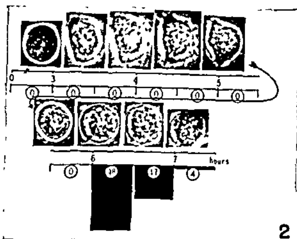
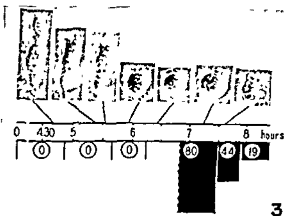
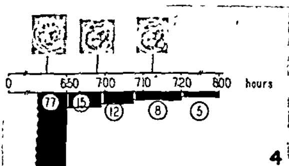


FIGURE 1. Growth curves of poliomyelitis virus type 1, Brunhilde strain, on four isolated monkey kidney cells. Abscissa: time after dilution of infected cells into nutrient medium. Ordinate: number of plaque-forming particles released by the cell. Arrows: time of vacuolization of hyaline zone (cf. FIGURES 3 and 4).







FIGURES 2, 3, and 4 Correlation between morphological changes observed in isolated monkey kidney cells after infection with poliomyelitis virus type 1, Brunhilde strain, and the release of virus FIGURES 3 and 4 correspond to the cells 3 and 4 in FIGURE 1 The numbers inside the circles represent the plaque forming particles released at various time intervals

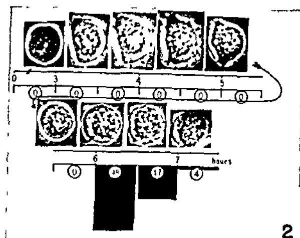
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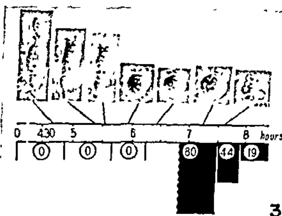
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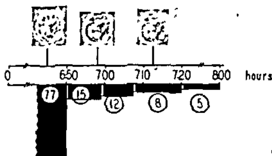
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FIGURES 2, 3 and 4 Correlation between morphological changes observed in isolated monkey kidney cells after infection with poliomyelitis virus type 1, Brunhilde strain, and the release of virus FIGURES 3 and 4 correspond to the cells 3 and 4 in FIGURE 1 The numbers inside the circles represent the plaque forming particles released at various time intervals

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Part II. Susceptibility of Cells and Organisms to Poliomyelitis

COMPARATIVE SUSCEPTIBILITY OF CELLS OF THE SAME TYPE TO INFECTION BY POLIOMYELITIS VIRUS*

By William F Scherer

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The generation of progeny different in certain properties from the parents is a universal biologic phenomenon. In tissue cultures, however, few variants of animal cells have as yet been recognized. Only two properties of cells, *i e*, morphology and malignancy have been reported to vary after prolonged serial cultivation *in vitro*.[1-5] Another measure of cell variation is the ability to propagate viruses. With more widespread use of animal cell strains for virus studies, cells with altered viral range may arise and be recognized

Of the many types of animal cells now under continuous cultivation *in vitro*, two strains have been studied extensively for their susceptibility to viruses The first was the L strain of mouse cells (Earle) derived originally from subcutaneous tissue and, later, from a single cell These cells are known to support growth of the viruses of herpes simplex, pseudorabies,[6] lymphocytic choriomeningitis, pseudolymphocytic choriomeningitis, encephalomyocarditis, MM strain,[7] western equine encephalomyelitis,[8] and eastern equine encephalomyelitis [9] The second strain of cells explored for its viral range was the HeLa strain of human epithelial cells (Gey) derived from a carcinoma of the cervix. A number of viruses are known to propagate in these cells, *i e*, poliomyelitis,[10] Coxsackie (Group B),[11, 12] herpes simplex, pseudorabies, vaccinia,[13] the encephalitis viruses of the eastern, western, West Nile, St Louis, and Japanese B types,[14] pseudolymphocytic choriomeningitis,[15] acute respiratory disease,[16, 17] mumps, and Newcastle disease [18]

The extensive use of strain HeLa cells for studies of poliomyelitis virus has resulted in the production of many generations of cells The chance of obtaining variants should therefore be greater for strain HeLa cells than for other cells less extensively reproduced *in vitro* Moreover, since the susceptibility of strain HeLa cells to certain viruses can be tested relatively easily, a continuing effort seems justified to identify variants by testing their viral range

Cells apparently in this category appeared about one year ago at Microbiological Associates, Inc, Bethesda, Md (MA), a company in mass production of strain HeLa cultures † At first, reports from various poliomyelitis laboratories indicated an abnormal response of the cells to poliomyelitis virus There- [illegible]

including their viral range, morphology, and growth rates.

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc , New York, N Y

† Promptly thereafter this company obtained a new stock of strain HeLa cells from our laboratory as a source for supply of cultures to customers

Materials and Methods

The procedures employed for cells from MA or for lines derived from them were identical to the methods described for cultivation of strain HeLa cells and for propagation of viruses in strain HeLa[10, 19] with the exception that, in the past six months, 5 rather than 10 per cent chicken serum in maintenance solution[4, 19] has been employed for virus studies It is noteworthy that the cells from MA attach less firmly to each other than strain HeLa cells, so that trypsin is not as regularly needed to disperse them

Results

During February 1954, cells were received at the University of Minnesota in four shipments from MA and were studied for their susceptibility to the viruses of poliomyelitis, Coxsackie (Group B), herpes simplex, vaccinia, and eastern equine encephalomyelitis (EEE) Two lines of cells, *i e*, lines 2 and 3, were subsequently derived from cells in two of the shipments The virus susceptibility of line 2 was studied after approximately 5 and 10 months cultivation in this laboratory Growth of line 3 has been too slow to provide sufficient cells for extensive viral studies

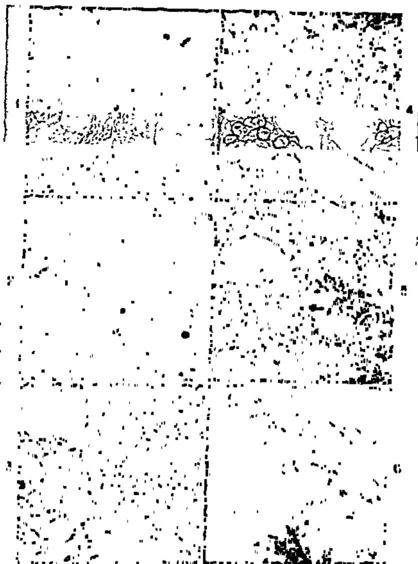
The information resulting from these studies will be presented chronologically in three categories (1) Initial observations with cells received from MA, (2) a historical account of cells in the two lines developed from cultures sent by MA, (3) response to viruses of cells in line 2 in July 1954 and in November-December 1954

Cells from Microbiological Associates, Inc In the spring of 1953, MA undertook mass production of strain HeLa cells This firm obtained cells from several laboratories, including ours By late fall 1953, several poliomyelitis laboratories reported failure to detect poliomyelitis virus with these cells as readily as with strain HeLa cells Therefore, in February 1954, four shipments [illegible]

size

Shipment 1 (February 1-2, 1954) Cells were sent with fluid of unknown composition and without fluid A mixture of human serum, 40 per cent, and Hanks' balanced salt solution, 60 per cent (HuS-40, H-60), was added to the tube cultures sent without medium Virus was added to cultures sent with the medium of unknown composition and to cultures rinsed thrice with balanced salt solution to remove viral inhibitors from the human serum medium. A [illegible]

four days



FIGURES 1, 2 and 3 Normal strain HeLa cells photographed one or two days after replacement of HuS-40, H-60 medium at 30° C ×150

FIGURES 4 and 5 Cells from line 2 in the 15th and 14th passages (TABLE II), in HuS-40, H-60 medium at 30° C. ×150

FIGURE 6 Cells from line 3 in the fourth passage (TABLE I), one day after renewal of HuS-40, H-60 medium at 30° C ×150

The microscopic appearance of cells from the fourth passage of line 3 is shown in FIGURE 6. Despite a renewal of fresh medium one day before the cells were photographed, many cells were round and clumped, and the cell membranes were not as extended or delicate peripherally as were the membranes of strain HeLa

TABLE 2
HISTORY OF LINE-2 CELLS FROM SHIPMENT RECEIVED FEBRUARY 18, 1954

Date	Passage	From	To	Medium[1] change	Spontaneous degeneration	Subline
2/24	1	3, TT[2]	1, PF	semiweekly		
3/5	2	1, PF	2, PF	"	4/9–5/4/54	6/29–2F
3/19	3	1, PF	2, PF	"	4/9–5/4/54	6/25–2C
4/3	4	1, PF	2, PF	"	4/20–5/4/54	6/25–2D
4/13	5	1, PF	2, PF	"		6/25–2E
6/4–12/22	6–17	1, PF	2, PF	"		

[1] Medium = HoS 40 H-60
[2] Cells were transferred from 3 test tube cultures (TT) received February 18, 1954, in HoS 10, BAFUF-5, MS-85, which was replaced by HuS 40, H-60 on February 23

cells (FIGURES 1 to 3) By two or three days after replacement of medium, the membranes of line-3 cells usually retracted, and the cells became round

Recently, by renewal of medium three times a week and by occasional addition of egg ultrafiltrate, the growth rate of line-3 cells has been increased, so that sheets of cells cover the surface of a Porter flask Thus, sufficient cells may soon be available for studies of virus susceptibility

[illegible]

required transfer approximately every two weeks Spontaneous degeneration of scattered cells in line 2 was observed in April and May 1954 Since then, cells in line 2 have stabilized in morphology (FIGURES 4 and 5), although recently it has been necessary [illegible]

for other work Cells in sublines 2C, 2E, and 2F have to date been transferred 10, 11, and 8 times respectively from the passage of origin in line 2 Transfer without difficulty at regular intervals has been possible Subline 2F has been maintained in both Porter flasks and in square 200 ml screw-capped bottles

[illegible] of strain HeLa (FIGURES 1 to 3 and 1?)

Cells from sublines 2C, 2E and 2F preserve at −60 to −70° C, as strain HeLa does [illegible]

the cells [illegible]



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3/5	2	1, PF	2, PF	"	4/9–5/4/54	6/29–2F
3/19	3	1, PF	2, PF	"	4/9–5/4/54	6/25–2C
4/3	4	1, PF	2, PF	"	4/20–5/4/54	6/25–2D
4/13	5	1, PF	2, PF	"		6/25–2E
6/4–12/22	6–17	1, PF	2, PF	"		

[1] Medium = HoS 40, H-60

[2] Cells were transferred from 3 test tube cultures (TT) received February 18, 1954 in HoS 10, BAFUF 5, MS-85 which was replaced by HoS 40, H 60 on February 23

cells (FIGURES 1 to 3). By two or three days after replacement of medium, the membranes of line-3 cells usually retracted, and the cells became round.

Recently, by renewal of medium three times a week and by occasional addition of egg ultrafiltrate, the growth rate of line-3 cells has been increased, so that sheets of cells cover the surface of a Porter flask. Thus, sufficient cells may soon be available for studies of virus susceptibility.

Information pertaining to the line of cells developed from shipment 3, line 2, and to four sublines, 2C–2F, is presented in TABLE 2. In contrast to the cells of line 3, cells in line 2 have reproduced readily, at a rate equal to that of strain HeLa cells. Despite frequent incubation at 30° C, cells became crowded and required *transfer approximately every two weeks. Spontaneous degeneration* of scattered cells in line 2 was observed in April and May 1954. Since then,

In June 1954, four sublines were started from different passage levels of line 2 (TABLE 2). After several passages, subline 2D was discontinued to make time for other work. Cells in sublines 2C, 2E, and 2F have to date been transferred 10, 11, and 8 times respectively from the passage of origin in line 2. Transfer without difficulty at regular intervals has been possible. Subline 2F has been

Cells from sublines 2C, 2E and 2F preserve at −60 to −70° C, as strain HeLa does,[20] for at least four months (August 1954 to January 1955).

In summary, two lines of cells were derived from cultures received from MA. One line has been readily propagated, the other has reproduced very slowly. Cells in both lines have remained distinguishable from strain HeLa cells in that the cells are usually smaller, they are less cohesive (and thus can be more easily dispersed), and they are less resistant to nutritional inadequacies and related low pH (i.e., 6.6 to 6.9). It must be emphasized, however, that *sheets of con-*



...from sublines 2C and 2E of line were infected by viruses on two occasions (TABLE 3). In July 1954, cells after

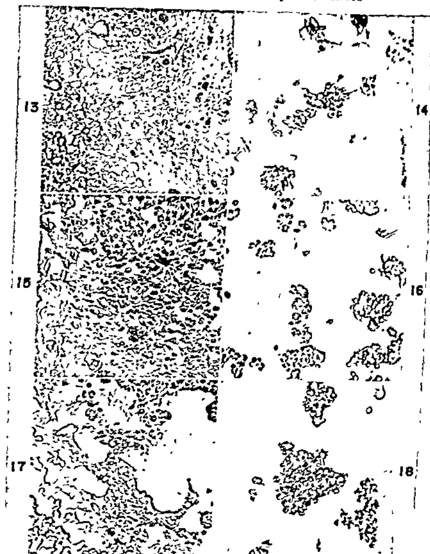
TABLE 3

COMPARATIVE VIRUS SUSCEPTIBILITIES AND YIELDS OF STRAIN HELA, AND LINES 2C, 2E AND 2F

	Virus inocula	CP[1] 2C	Yield of virus[2] 2C	Yield of virus[2] HeLa	CP[1] 2E	Yield of virus[2] 2E	Yield of virus[2] HeLa	CP[1] 2F	Yield of virus[2] 2F	Yield of virus[2] HeLa
Poliomyelitis										
7/54 T1	1,000,000 $TCID_{50}$	=			=					
	1,000 "	≠[3]			≠					
T2	500,000 "	=			=					
	500 "	≠[3]			≠					
T3	320,000 "	=			=					
	320 "	≠[3]			≠					
11-12/54 T3	1,000 "							=	5 5	5 0
	320 "	=	4 7	5 7	=	5 7	5 7			
	10 "							=	7 0	6 5
Herpes simplex										
7/54	MB + HeLa[1]	=			=					
11-12/54	Undil MB (MB[6] + HeLa[16] + MB[3])[4]							=	3 5	4 0
Vaccinia										
7/54	CAM[7] + HeLa[1] + CAM[1] + HeLa[1]	=			=					
11-12/54	10^2 dil CAM[7] + HeLa[1] + CAM[1]							=	3 5	4 5
EEE										
11-12/54	Undil MB[3]	=			=			=		

a total of six passages in this laboratory (subline 2C) and eight passages (subline 2E) were inoculated with the viruses of poliomyelitis, herpes simplex, and vaccinia Strain HeLa cell cultures were inoculated in parallel The responses to herpes simplex and vaccinia viruses of sublines 2C and 2E were indistinguishable from that of strain HeLa (TABLE 3) Poliomyelitis virus, in large quantity, rapidly destroyed these cells With smaller quantities of poliomyelitis virus, however, the cytologic response was abnormally slow Stages of cellular destruction comparable to those seen in control strain HeLa cultures occurred about one day later in cultures of sublines 2C and 2E

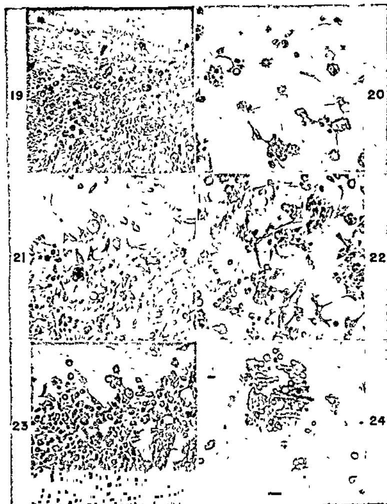
In November and December 1954, cells from sublines 2C and 2E, after 12 and 15 passages respectively, were again inoculated with poliomyelitis virus, in relatively small quantity (TABLE 3) On this occasion, they responded rapidly and comparably to strain HeLa cells (FIGURES 13 to 18) Furthermore, at this time, the response of sublines 2C and 2E to EEE virus was like that of strain HeLa (FIGURES 19 to 24)



FIGURES 13, 15, and 17. Normal strain HeLa, subline 2C and subline 2E cells in test tube cultures as controls for cells shown in FIGURES 14, 16, and 18. Photographed three days after rinsing and adding ChS 5, MS-95 at 36° C. ×75.

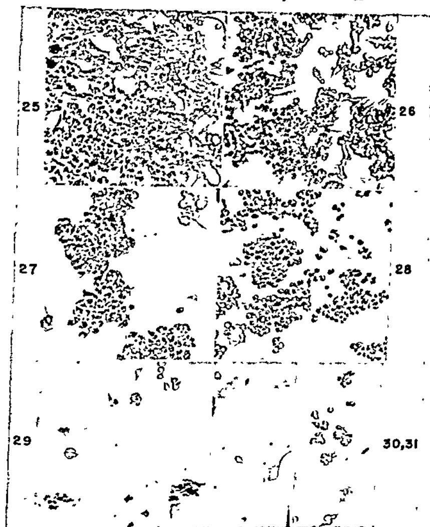
FIGURES 14, 16, and 18. Effects of poliomyelitis virus upon strain HeLa, subline 2C and subline 2E respectively, three days after inoculation of type 3 virus, Saukett strain in ChS-5, MS 95 at 36° C. ×75.

Cells from subline 2F, after a total of nine passages, were studied in November and December 1954 for their response to the viruses of poliomyelitis, herpes simplex, vaccinia, and EEE. The cytologic response of the cells to these viruses was similar to that of strain HeLa (TABLE 3, FIGURES 25 to 30 and 32 to 37). Moreover, the yields of poliomyelitis and herpes simplex viruses from subline



2Γ cells were comparable to yields from parallel cultures of strain HeLa cells (TABLE 3). The slightly lower yield of vaccinia virus from subline 2Γ cells (TABLE 3) will require confirmation to be significant.

During the experiments with subline 2Γ cells, degeneration occurred in control cultures, rinsed thrice with balanced salt solution and incubated for five



[illegible]

to seven days at 36° C in ChS-5, MS-95 This spontaneous degeneration made it difficult to evaluate the end points of virus titrations in subline 2F cells Nevertheless, an attempt was made to compare the sensitivity of subline 2F and of strain HeLa cells for quantitation of poliomyelitis, herpes simplex, and vaccinia viruses As shown in TABLE 4, the titration end point for poliomyelitis



virus in subline 2F cells was essentially equal to that in strain HeLa cells The end points of herpes simplex and vaccinia viruses in subline 2F cells, however, were indeterminate because of spontaneous cellular degeneration

In another experiment, an attempt was made to isolate an infectious agent from cultures showing spontaneous degeneration of line 2F cells (FIGURE 31) Since there was no microscopic evidence of bacteria or fungi in the cultures,

TABLE 4

VIRUS TITRATIONS IN STRAIN HELA AND LINE 2F

Virus	HeLa	2F Cells
	Neg log $TCID_{50}$	Neg log $TCID_{50}$
[illegible] T3 HeLa [illegible]	5 2	4 7 (based on total degeneration, difficult to read because of spontaneous degeneration)
[illegible] MS – 1 HeLa	3 7	Indeterminate because of spontaneous degeneration
Vaccinia CAM – 1 HeLa	3 7	Indeterminate because of spontaneous degeneration 100% deg through 10^{3}

[illegible] chorioallantoic membrane (CAM) suspensions passed once in strain HeLa

the cells [illegible] on the glass and the supernatant fluid from two test tube cultures were [illegible] and inoculated intracerebrally into five white mice three weeks of age, and into two test-tube cultures of strain HeLa cells, after [illegible] CSS-5, MS-93 No signs of illness became evident in the mice, and no progressive cellular destruction of strain HeLa cells occurred over a [illegible] period. Thus, this spontaneous degeneration is as yet unexplained.

In summary, after 9 to 15 passages and 10 months cultivation in this laboratory, cells derived from one shipment of cultures responded to poliomyelitis virus as strain HeLa cells do. Moreover, these cells produced poliomyelitis virus in amounts comparable to the yields from strain HeLa. Although the cytologic response of the cells to vaccinia virus in February 1954, on arrival from MA was somewhat atypical on one occasion, the cytopathogenic effect of this virus for these cells in July 1954 and in November and December 1954 was like that for strain HeLa cells. The cytologic response of the cells to herpes simplex and EEE virus has remained like that of strain HeLa.

Discussion

Experiments started in February 1954 to study cells from MA have resulted in several observations (a) On arrival in this laboratory, the cells were smaller than strain HeLa cells and responded atypically to poliomyelitis, Coxsackie, and possibly vaccinia viruses but typically to herpes simplex and EEE viruses, (b) cells derived from one shipment, line 2, have been propagated readily through 17 passages in medium of the same composition employed for strain HeLa cells, i.e. a mixture of human serum, 40 per cent, and balanced salt solu- rough
tion, 60 per cent, cells from another shipment have reproduced slo r 1954 (although they
only six passages between February and Dec ent change o and
have been stimulated to more rapid growt lls in the lin lti-
by occasional incorporation of egg ultrafilt smaller, ve,
vation in this laboratory have remained o pH tha
and less resistant to nutritional inadequa , surviv
cells—yet the cells from one line, like those line
storage for at least four mon s; (d) cells in and
as strain HeLa does t is, herpes

Fundamental to this study is a question as yet unanswered unequivocally, *i e*, are the cells received from MA and employed in these studies derivatives of strain HeLa cells? Since the origin of these cells was not the result of a carefully observed experiment, it may be impossible ever to answer this question with certainty At this time, it seems best to present the available evidence for and against this relationship and let the reader form a conclusion if he desires to do so

Affirmative evidence for derivation of these cells from strain HeLa can be itemized as follows

(1) the cells form [illegible] cells and [illegible] in some [illegible]

[illegible] strain HeLa cells, but requires incorporation of tissue extract for growth of many other cells

(3) Cells of one line, after 9 to 15 passages and 10 months cultivation in this laboratory, respond to the viruses of poliomyelitis, herpes simplex, vaccinia, and EEE as strain HeLa does The other line has yet to be tested

(4) The cells throughout these studies have responded as strain HeLa cells do to herpes simplex and EEE viruses

(5) Freezing and storage of the cells from one line for four months has been successful by employing the methods used for strain HeLa cells

(6) Although MA in the spring of 1953, obtained "strain HeLa cells" from several laboratories, the firm claims that the cells received for this study came originally from a single bottle culture of strain HeLa supplied from our laboratory in June 1953

(7) The personnel of MA state that at no time prior to shipment of cells to the University of Minnesota was there any other strain of human or animal cells in their laboratory, and that the chicken embryonic extract and bovine amniotic fluid ultrafiltrate employed for cultivation of these cells was filtered through Selas 0 03 porosity filters to sterilize and eliminate cells

Evidence against a relationship between these cells and strain HeLa cells includes (1) the origination of these cells under uncontrolled experimental [illegible] when the pH has reached 6 6–6 8, (3) the abnormal cytologic response to poliomyelitis and Coxsackie viruses in February 1954 and to small inocula of poliomyelitis virus in July 1954, after six to eight passages and five months' cultivation in this laboratory

Immunologic methods may assist in clarifying this relationship An attempt is underway to prepare antibodies against strain HeLa cells and line 2 cells

If these cells came from strain HeLa cells, their mode of origin is unknown Whether use of media other than HuS-40, H-60 or of animal sera or body fluids such as horse serum could have been a causative factor in their development is impossible to determine in retrospect

COMPARISON OF SUSCEPTIBLE AND RESISTANT CELLS TO INFECTION WITH POLIOMYELITIS VIRUS*

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Ever since the discovery of the virus by Landsteiner and Popper,[1] poliovirus has manifested an extremely narrow host range. Even *in vitro* strains of the three virus types have been found to multiply and cause cytopathic changes in cultures of tissue derived from primate sources only. A wide variety of tissues of primate origin are susceptible to the action of poliovirus,[2] but human kidney tissue,[3] as well as that from rhesus and cynomolgus monkeys,[4] seems to be more susceptible than most of the normal tissues employed for virus growth. The results of studies on the susceptibility of tissues from various species have been obtained either with tissue fragments embedded in a plasma clot, or with suspended tissue fragment cultures Since monolayer cultures of renal epithelium are more susceptible to poliovirus, we considered it possible that such cultures obtained by trypsinization[5, 6] from various species might prove more susceptible than hitherto suspected The purpose of this report is to compare the reactions to poliovirus of epithelial cells derived from the kidneys of four different species

The animals selected for this study were newborn rabbits, immature hamsters, and capuchin and rhesus monkeys These animals show a progressively

When the monolayers had formed, each was inoculated with Brunhilde, Y-SK, and Leon strains of poliovirus which had been through 25 passages in cultures of rhesus monkey kidney Only the rhesus kidney cells supported the growth of these polioviruses This is illustrated in TABLE 1 for the capuchin monkey The three representative strains of poliovirus were incubated with cultures of capuchin renal epithelial cells No cytopathic changes were noted and, after two weeks of incubation, the culture fluids were harvested and titrated in rhesus kidney cultures The results show that the three types of tissue-culture virus persisted without giving any evidence of having multiplied, since the titer of virus found did not differ significantly from that of the control tubes without tissue

Experiments with capuchin cultures inoculated with human stools proved to contain poliovirus suggest, however, that renal epithelial cells from this species may support the growth of type 1 poliovirus Capuchin cultures were inoculated with three different specimens of human stools, each stool containing a different virus type The fluids were harvested at the end of two weeks and titrated in rhesus kidney cultures As the results in TABLE 1 show, the fluids from the cultures of capuchin cells inoculated with type 1 fecal virus contained at least 10,000 times more virus than the control tubes without tissue Similar results were obtained on second passa e of this material in capuchin

* Aided by a grant from the National Foundation for Inf Inc , New York, N Y

TABLE 1
SUSCEPTIBILITY OF CAPUCHIN KIDNEY EPITHELIAL TISSUE CULTURES TO POLIOMYELITIS VIRUS

Virus*	Type	Tissue	1st passage in capuchin TC Cytopath	Result of titration in rhesus kidney TC†		2nd passage in capuchin TC Cytopath	Result of titration in rhesus kidney TC†	
				Cebus	Control (no tissue)		Cebus	Control‡
Standard								
Brun	1	capuchin	—	4 0	3 5			
Y-SK	2		—	3 0	2 5			
Leon	3		—	4 0	3 0			
Stools								
E-15	1	capuchin	—	4 0	0	—	3 1	0
E-21	2		—	0	0	—	0	0
E-4	3		—	0	0	—	0	0
E 15	1	rhesus	+					
E-21	2		+					
E-4	3		+					

* Undiluted
† Negative log per 0 1 ml inoculum, 0 means that virus was not detectable in undiluted or 10 times diluted inocula
‡ Dead tissue

cultures The other stool specimens containing types 1 and 2 polioviruses did not persist either in the tubes with or without capuchin cells This observation is in keeping with the findings made in this laboratory 12 years ago[7] that, while rhesus adapted poliovirus inoculated intracerebrally failed to infect capuchin monkeys, virus obtained from human patients was pathogenic and readily produced paralysis A correlation may also exist between our observation and that of Jungeblut and de Rodaniche,[8] who reported that spider monkeys, which are also New World monkeys, are susceptible to the intracerebral inoculation of type 1 poliovirus but not to types 2 or 3

Since the kidney cells from rabbits, hamsters, and capuchin monkeys proved to be nonsusceptible to tissue-culture virus, attempts were made to alter their resistance One approach to this problem was to grow the renal cells of the resistant animals together in such intimate contact with the susceptible cells of the rhesus monkeys that, except for the hamster cells, they could not be told apart microscopically After the cells had grown together, virus was added and cytopathic changes occurred, presumably only in the susceptible cells, and the assumption was made that the unaffected cells were those derived from the resistant species These cultures had been prepared by seeding tubes with varying concentrations of susceptible and nonsusceptible cells The fluids, harvested after inoculation with virus of cultures seeded with an equal number of both types of cells, were passed in cultures containing fewer and fewer of the susceptible cells until the final passage, which was carried out in cultures containing cells derived from the kidneys of the resistant animals only Titrations of the fluids from these cultures showed that virus multiplication in the nonsusceptible cells had not occurred Similar results have been obtained by others[3, 9]

A second method of inducing the change from resistance to susceptibility was tried Three-week-old hamsters were given two intramuscular inoculations of cortisone 24 hours apart. Two hours after the second inoculation, half of these animals were subjected to X-ray irradiation; another group of hamsters, not treated with cortisone, was also irradiated. The animals were sacrificed two and four days later, their kidneys removed, and monolayer cultures prepared in tubes. When these cultures had grown out, they were inoculated either with Y-SK virus passed 25 times in tissue culture, or with MEF1 virus passed intracerebrally in hamsters None of the four groups of cultures supported the multiplication of either virus.

The reason for the lack of susceptibility was explored. The possibility was considered that the primary step in virus infection, namely, virus adsorption might be involved, since it is known that bacteriophage may be adsorbed by but not multiply in some nonsusceptible cells. Experiments were therefore carried out to determine whether the lack of susceptibility might be associated with the lack of adsorptive capacity for the virus. Renal epithelial cells of rhesus and capuchin monkeys, normal hamsters, and hamsters treated with cortisone and with X-ray irradiation were grown in Petri dishes, and their capacity to adsorb Y-SK virus was determined by the plaque technique of Dulbecco and Vogt.[5] Virus was added to the renal epithelial cells, which were

to the plates The removed virus suspension and washings were combined and tested for the number of infectious virus particles in fresh plates TABLE 2 illustrates the results obtained with rhesus and capuchin monkeys After 10 minutes of incubation, an average of 14 plaques were adsorbed This average is 12 per cent of the total virus added. The unadsorbed virus and the washings, which constitute the free virus, yielded an average of 16 5 plaques When the dilution factor was taken into consideration, the total amount of free virus was calculated to be 125 The amount of virus adsorbed, calculated by considering the total virus as the adsorbed plus the free, turned out to be 11 per cent, a good correlation with the percentage of virus as calculated above from the total number of plaque-forming particles added By 90 minutes, the rhesus cells had adsorbed 79 per cent of the virus added

In contrast to the rhesus cells, the capuchin cells failed to adsorb a measurable amount of virus, since all of the added virus was recovered, even after an incubation period of 90 minutes In experiments carried out in the same way, *the renal cells from all the other animals, including the treated hamsters,* failed to adsorb virus It should be pointed out that the lack of virus adsorption by these resistant species, particularly the capuchin monkeys, does not preclude the possibility that, if the virus were to gain entrance into the cells by some other means, it might still multiply there

In addition to these studies on the susceptibility and resistance of renal cells from different species, a comparative study has been made of the infection by poliovirus of monkey kidney cells *in vivo* and *in vitro* The monkeys were inoculated directly into their kidneys with Y-SK tissue culture virus At ap-

TABLE 2

ADSORPTION OF Y-SK VIRUS TO RHESUS AND TO CAPUCHIN KIDNEY EPITHELIAL CELLS

Time (min)	Adsorbed no. of plaques on original plate		Free no. of plaques		Fraction adsorbed (per cent)	
	10^{-4}	10^{-4}	Observed	× Dilution factor 7.6	A	B
Rhesus						
10	14		16.5	125.4	12	11
15	18.5				15	
30	21.5		13.5	102.6	20	19
60		5			42	
90		9.5			79	
Capuchin						
30	—		15	114.0		0
90	—		19	144.4		0
Control		12				

Results are averages of duplicate samples

$$A = \frac{\text{Adsorbed}}{\text{Total virus added}} \qquad B = \frac{\text{Adsorbed}}{\text{Adsorbed} + \text{Free}}$$

TABLE 3

SUSCEPTIBILITY OF RHESUS KIDNEY CELLS IN VIVO AND IN VITRO

Day	Recovery titer expected*	Titer found*	Recovery per cent	Virus in cultures	Neutralizing antibodies
1	3.7	3.3	40	+	
2	4.5	<1.0	<0.03	+	
3	4.5	2.0	0.31	+	
4	4.5	1.5	0.10	+	
7	4.5	<1.0	<0.03	−	1:10
10	3.7	1.6	0.80	+	1:20

* Negative log per 0.1 ml. inoculum

propriate intervals after inoculation, the animals were sacrificed and the kidneys removed and treated as follows: one kidney was homogenized in a Waring blendor, and the amount of virus present in the cell-free suspension determined, the second kidney was trypsinized and cultures prepared in tubes.

As the results in TABLE 3 show, the amount of virus present in rhesus monkey kidneys decreased 40 per cent in the first 24 hours after inoculation and, by 10 days, less than 1 per cent of the virus remained. The cells from the second kidney grew out in tissue culture into monolayers and degenerated spontaneously in four to six days. Two further facts of interest emerge from these results as well: (1) virus could be detected in tissue culture even when none was detectable in the cell-free homogenate, and (2) the presence of antibodies in the serum of the monkey sacrificed at seven days indicated that virus had been

present, although the amount remaining in the kidney was so small that it could not be detected even in the highly susceptible renal epithelial cells in the culture tubes.

This experiment was repeated with cynomolgus monkeys, one group being treated with cortisone. The amount of virus present in the monkey kidneys *in vivo* decreased, so that, by the ninth day, no evidence of [illegible] ation

No

eated

cynomolgus monkeys, nor between this species and rhesus monkeys. It is

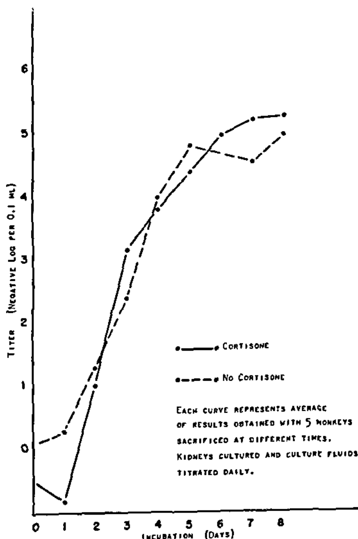


FIGURE 1. Titration of fluids from cultures of cynomolgus monkeys inoculated in vivo with virus

noteworthy that, even though one of the monkeys came down on the ninth day with paralytic disease, no evidence of virus multiplication in the kidney could be obtained. This lack of virus multiplication in the kidney is comparable with the observations of Evans *et al.*[10] and Ledinko *et al.*,[11] who found also no evidence of virus multiplication after intratesticular virus inoculation.

The fluids from cultures of kidneys that had been inoculated *in vivo* and subsequently used to initiate the cultures were collected daily and titrated. The results are illustrated in FIGURE 1. These curves represent the averages of individual curves obtained by the daily titration of the fluids from the cultures prepared on the day a given animal was sacrificed. As these curves demonstrate, virus was barely detectable, and sometimes not at all on the first day of incubation *in vitro*, but it multiplied considerably, so that by the eighth day of incubation, the tissue culture fluids had an average titer of over 10^{-5} per 0.1 ml.

Similar experiments were carried out with *Cebus capucina*, the New World capuchin monkey. A comparison of the multiplication of virus *in vivo* between the Old and New World monkeys, as illustrated in FIGURE 2, shows that the kidneys of the capuchin monkeys and the kidneys of the cynomolgus monkeys respond the same way *in vivo*. There was a steady decrease in the amount of virus in the kidneys of the living animals whether or not they had been treated with cortisone. The amount of virus found in the kidneys of the

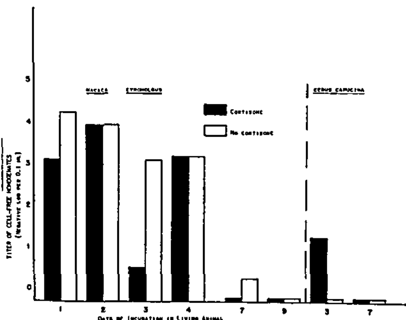


FIGURE 2. Comparison of virus multiplication *in vivo* between Old and New World monkeys.

capuchin monkeys at the end of three and seven days correspond approximately to that found in the kidneys of the cynomolgus monkeys for the same periods. Thus, both the cynomolgus and capuchin monkeys are approximately equally resistant to the *in vivo* inoculation of virus.

The results obtained *in vitro*, however, are in marked contrast to those obtained *in vivo*. As the curves in FIGURE 3 show, the renal epithelial cells derived from the *in vivo* inoculated cynomolgus monkeys are highly susceptible *in vitro*. This curve is an average of daily titrations of fluids from cultures grown from the kidneys of eight monkeys sacrificed at the time intervals indicated in FIGURE 2. Is this

cells of these animals, which remain resistant *in vitro*.

Finally, an experiment was carried out to determine whether the inoculated virus might have penetrated the kidney cells of the susceptible animals inocu-

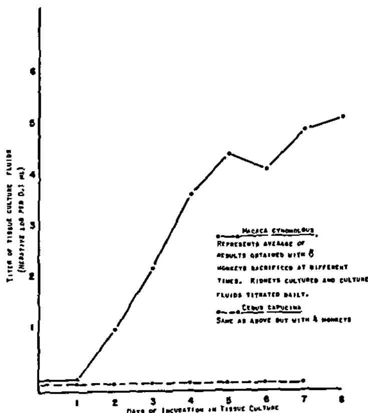


FIGURE 3. Comparison of virus multiplication *in vitro* between Old and New World monkeys. These curves represent the averages of daily titrations of fluids from cultures grown from the kidneys of eight cynomolgus and four capuchin monkeys sacrificed at the time intervals indicated in FIGURE 2.

TABLE 4

DETERMINATION OF LOCATION AND AMOUNT OF VIRUS ON CELLS DERIVED FROM INOCULATED RHESUS KIDNEY

Dilution	Plaques	Average	Calculated multiplicity
I. Intact cells			
Original	28, 26, 16	23.3	$\frac{2.33 \times 10^{1}}{1.1 \times 10^{5}} = \frac{2.1 \text{ Virus particles}}{10{,}000 \text{ Cells}}$
1/10	3, 2	2.5	
II. Supernate of disrupted cells			
Original	41, 38	39.5	$\frac{3.95 \times 10^{1}}{1.1 \times 10^{5}} = \frac{3.6 \text{ Virus particles}}{10{,}000 \text{ Cells}}$
1/10	2, 4, 3	3	

lated *in vivo* and might have grown in them to a limited extent, or whether the virus might simply have been free extracellularly in the kidney *in vivo* and might have been adsorbed to the cells only during the trypsinization procedure. Cells from the kidney of a rhesus monkey inoculated 24 hours previously were plated by the plaque method to determine the number of infectious centers. As shown in TABLE 4, an average of 23 plaques was counted. A portion of cells from the same suspension was disrupted by homogenization, and the supernatant fluid also titrated in plates. This yielded an average of 40 plaques, an approximate twofold increase which can hardly be considered significant if virus were growing in some of the kidney cells *in vivo*. While this experiment

particles in the suspension of cells derived from the kidney of the *in vivo* inoculated monkey. As illustrated, there were 2.1 to 3.6 virus particles per 10,000 cells, emphasizing again the highly susceptible nature of the renal epithelial cells *in vitro*.

These observations may be summarized as follows:

(1) Poliovirus passed 25 times through rhesus monkey kidney cultures did not multiply in renal epithelial cells derived from newborn rabbits, immature hamsters, and capuchin monkeys. Experiments with capuchin cultures inoculated with human stools suggest, however, that the renal cells from this species may support the growth of type 1 poliovirus derived from human sources.

(2) Induction of the change from resistance to susceptibility attempted by growing nonsusceptible and susceptible cells together and by treatment of hamsters with cortisone and X-ray irradiation did not succeed.

(3) The lack of susceptibility of the cells tested might be due to their lack of capacity to adsorb Y-SK virus, for it was found that while the susceptible rhesus cells adsorbed about 80 per cent of this virus in 90 minutes, the resistant cells did not adsorb a measurable amount.

(4) A comparison of the response to poliovirus by kidney cells *in vivo* and *in vitro* showed that renal cells from rhesus or cynomolgus monkeys proved to be resistant to tissue-culture adapted virus when inoculated *in vivo*, but were completely susceptible *in vitro*. This finding is in contrast to the cells of capuchin monkeys which were resistant *in vitro* as well as *in vivo*.

(5) The extraordinary *in vitro* sensitivity of the susceptible kidney cells from animals inoculated *in vivo* was shown also by the fact that, in several instances when no virus could be detected in cell-free homogenates, virus could be usually detected in cultures made from the other kidneys of these animals, and by the fact that calculations made from an experiment carried out by the plaque technique showed that there were only 2.1 to 3 6 infectious virus particles per 10,000 cells, but when this suspension of cells was grown out in tubes, a high yield of virus was produced

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Discussion of the Paper

DOCTOR M M SIGEL, *Department of Health, Education, and Welfare, United States Public Health Service, Montgomery, Ala* . Heretofore, problems of resistance at the cell level in the area of host-virus interaction have been virtually ignored Closer scrutiny of the workings of susceptibility and resistance of

of Carver Foundation HeLa cells This line, designated 95, has retained this characteristic up to this time The cells have undergone 34 weekly passages The resistance appears to be selective in nature The cells are relatively re- [illegible]

One reverted to normal susceptibility to poliomyelitis virus by the second subpassage The other, after 24 weekly passages, has remained at the same level of resistance as its parent

With respect to Doctor Kaplan's remarks about his failure to alter the resistance of cells by prior treatment of the intact animal with cortisone, I should like to state that we have obtained evidence that cortisone is capable of modifying susceptibility of one type of cell if added to the cells in tissue culture In fact, a rather curious situation has been discovered Cortisone was found to cause increased resistance of HeLa cells to poliomyelitis virus-induced cytopathogenic changes when given at the time of infection with the virus On the other hand, an increased susceptibility was obtained when the hormone was introduced into the cultures at the time of initiation of cell culture and then washed away prior to infection with virus

SOME COMPARATIVE OBSERVATIONS ON THE BEHAVIOR OF POLIOMYELITIS VIRUSES IN ANIMALS AND IN TISSUE CULTURE*

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During the past two years, the Virus Laboratory in the University of Kansas has been engaged in studies designed to obtain strains of poliomyelitis viruses modified in regard to their paralytogenic properties for animals commonly used in experimental poliomyelitis At the beginning of the study, it was realized that essential information about some of the biologic characters of poliomyelitis viruses was needed There were available many strains of poliomyelitis viruses that had been studied in some detail in the typing program.[1] Among these, we selected 24 strains for comparative studies in animals and in tissue culture prior to experimental approaches designed for "purification" and search for mutants in the virus population A part of these comparative studies provide some of the data reported here I have also appended some comparative data on the usefulness of tissue culture methods for the detection of virus directly from human or animal sources, and for the assay of serum neutralizing antibodies

Materials and Methods

Poliomyelitis viruses The strains of poliomyelitis viruses studied are listed in the text. A pool of virus, prepared from the spinal cords of infected monkeys, was prepared with each strain [1] Following adaptation of each strain in tissue culture, each was passaged three or more times. Excepting the few strains difficult to adapt in tissue culture, the fourth passage was made in order to prepare a pool (100 ml) of virus Aliquots of each virus pool were distributed in 1 0 ml ampoules and stored in a dry-ice cabinet The brain and spinal cords obtained from paralyzed mice[2] were used to prepare additional virus pools The studies made are based on observations made with the reference pools of virus

Titration of virus pools Decimal or half-log dilutions of a reference pool of virus was made in medium 199 [3] Animals or tissue cultures were inoculated immediately, beginning with the tube containing the least amount of virus Four to eight tissue culture tubes, eight mice, and two to six monkeys were used to test each virus dilution Titrations were often repeated in tissue culture and in mice, but only infrequently in monkeys Results appearing in the text are cumulative, based on two or more titrations

Tissue cultures The methods used for the propagation of poliomyelitis viruses in several tissue culture systems have been described [4, 5, 6, 7] Observations reported here are based on tubes containing actively growing cells in

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0.9 ml of nutrient or maintenance solutions An 0.1 ml inoculum was delivered to the tissue cultures, excepting in the colorimetric test where 0.25 ml inoculum was used. Inoculated and control tubes were observed for a period of seven days

Animal tests. Monkeys (rhesus and cynomolgus) and Swiss mice were inoculated intracerebrally with reference pools of virus Monkeys received 0.5 ml, mice 0.03 ml of the appropriate dilution of virus The animals were examined daily for a period of 30 to 40 days The CNS of each monkey was examined for experimental lesions characteristically found in poliomyelitis Mice were scored on the basis of paralysis or death occurring on or after the second day after inoculation

Results

Variations encountered in tissue culture systems. It was recognized early that quantitative differences existed in tissue culture systems employed for replication of poliomyelitis viruses A difference was apparently related to lytic resistance of cells such as testicular fibroblasts used for virus replication. For example, virus pools prepared in testicular fibroblast cultures often, but not always, provided titers two to four logs lower in fibroblast than in kidney epithelial cell cultures Another difference was related to the adequacy of nutrient media used to initiate and maintain cultures of actively growing cells We have used several different media, as have other workers, more or less on the premise that they were deemed adequate without evidence in regard to performance level. It was recognized, however, that the addition of serum provided a milieu conducive to vigorous cell growth of kidney epithelial cells and then to a more abundant replication of virus within the cell mass For example, implants seeded in lactalbumin hydrolysate gave less regular and generally less vigorous growth of kidney epithelial cells than implants seeded in a medium consisting of Earle's solution, 70 per cent, acetone embryo extract, 10 per cent, and monkey serum, 20 per cent The latter medium initiated and maintained excellent cellular growth, and virus titers were two to three logs higher than those observed in cells nourished with lactalbumin hydrolysate Another difference was related to the cell mass introduced and is subject to the variables

adapted Lansing strain of poliomyelitis virus required seven consecutive passages in kidney epithelial cells before general lysis was observed Probably,

next passage level A TCD_{50} of $10^{-5.5}$ was obtained in kidney roller tubes, and an ID_{50} of $10^{-1.5}$ in mice at the eighth passage level A relative inability of virus to destroy the cell mass was found with two additional type 2 strains of poliomyelitis viruses In comparison, the 21 additional strains which we have

TABLE 1

COMPARATIVE DATA ON TITRATION OF EIGHT STRAINS OF POLIOMYELITIS VIRUSES IN SEV
TISSUE CULTURE SYSTEMS

Strain of virus	Type	TDC₅₀ obtained in several tissue culture systems							
		No serum		Serum present during cellular growth phase					
		Rh testis RT	Rh kidney LA RT	Rh kidney RT Earles + MS	Rh kidney 199 RT	Rh kidney 199 MI	HeLa ST	HeLa suspended cells	Human kidney
Brunhilde*	1	3 9	3 8	5 8	3 3?	—	<3 0	2 2	>6
Brooks	2	4 0	5 4	8 6	6 8	6 6	6 1	7 3	7
BEV-3	2	3 4	6 0	6 7	5 2	5 6	5 6	5 4	7
Fotovich	2	—	6 0	7 7	6 7	5 7	5 6	—	6
Linden	2	—	4 9	8 5	<7 0	5 3	5 6	6 2	<7
Orr	2	—	5 4	6 0	7 2	5.5	5 6	5 9	—
Y-SK	2	2 8	5 0	5 1	5 5	5.6	5 1	5 7	5
Saukett*	3	3 4	5 5	7 8	<5 0	—	—	—	5

RT = roller tube, LA = lactalbumin hydrolysate, MI = *metabolic inhibition*, ST = stationary tube, MS = monkey serum

* Pool prepared in Roux flasks in testicular implants all others from monkey kidney implants

studied promptly lysed kidney cells, and replication of virus in high titer wa
ac

sy.

data also appear in TABLE 1 I am indebted to Doctor Jerome Syverton fo the titration data obtained with HeLa cells and performed in his laborator The results were quite uniform, and they indicate that preference of any one c the three systems must be one of choice, since corresponding end points wer obtained with each of the eight strains of poliomyelitis viruses included in th test

One other variable affecting measurements, particularly in titration o viruses, was the *time of recording cell lysis* TCD_{50} *end points scored on th* third day were almost always one or two logs lower than end points scored o the seventh day Time was of some, although comparatively less, importanc in the behavior of most strains that had been passaged in tissue culture I the inoculum contained many infectious units (*e g* , 1000 TCD_{50}) replicatio was rapid, and maximal titers, based on growth-curve data, were often reached 24 to 36 hours after inoculation of actively growing cells After this time there was a slow change downward in TCD_{50} over a period of four days, indicating that, after maximal replication had been achieved, the rate of virus decay occurring during the period under the conditions of the experiment was quite slow Results obtained in studies with two strains of poliomyelitis viruses appear in TABLE 2

Comparative studies with established strains of viruses Another series of studies was made with reference pools of virus in order to compare the ID_{50} end point, using monkeys, mice, and kidney epithelial tissue cultures The reference pools consisted of monkey and of mouse CNS emulsions and fluids

TABLE 2

COMPARATIVE DATA ON FOUR STRAINS OF POLIOMYELITIS VIRUSES TITRATED AT INTERVALS IN HELA AND MONKEY KIDNEY EPITHELIAL CELL SYSTEMS

Time of harvest (hours)	In HeLa*		In monkey kidney†	
	Brunhilde	Brooks	Brunhilde	Brooks
Original	6 0	9 0	6 0	9 0
36	<6 0	5 0	6 3	7 5
60	8 5	7 0	5 8	7 0
84	7 5	—	5 6	6 5
108	6 5	6 5	5 0	5 7
132	6 5	—	5 1	5 0

* Virus grown in HeLa cells
† Virus grown in monkey kidney cells

TABLE 3

COMPARATIVE ID_{50} END POINT SCORES OBTAINED WITH REFERENCE POOLS OF POLIOMYELITIS VIRUSES IN MONKEYS, MICE, AND KIDNEY EPITHELIAL CELL CULTURES

Strain of virus	ID_{50} endpoint scores in								
	Monkeys			Mice			Tissue culture		
	Monkey CNS pool	Mouse CNS pool	TC* pool	Monkey CNS pool	Mouse CNS pool	TC pool	Monkey CNS pool	Mouse CNS pool	TC pool
Fotovich	5 7	6 1	7 0	0	5 2	3 8	6 5	8 2	8 0
Orr	5 8	>4 5	6 0	0	5 4	4 0	6 0	8 3	6 5
Lansing	Inc	4 0	4 5	—	5 4	4 2	3 7	<1 0	4 3
Y-SK	4 9	5 4	6 0	—	5 8	3 1	6 8	8 5	6 0

Inc = incomplete
Based on ID_{50}/gm or ml
* In trypsinized monkey kidney roller tubes

harvested from monkey kidney epithelial cells, all infected with several strains of poliomyelitis viruses A summary of the results of these studies appears in TABLE 3

The results may be considered both from observed and from certain theoretical aspects The virus reference pools, namely those derived from monkey CNS and mouse CNS and from tissue cultures provided infectivity titers of corresponding values when each was tested intracerebrally in monkeys Tissue culture virus gave somewhat higher end points, but the differences are not significant When the virus pools were assayed in mice, ID_{50} end points obtained were numerically larger with mouse-adapted than with tissue culture-adapted virus Assayed in monkey kidney epithelial cultures, the reference monkey CNS and tissue culture virus pools provided some irregular results The data suggest that these two reference pools probably have equivalent end points Mouse-adapted virus pools provided, with one exception, consistent and significant differences in ID_{50} scores obtained in monkeys, mice, and tissue culture

There was found an inverse relationship between the susceptibility of monkey kidney epithelial cells for mouse-adapted virus, and the susceptibility of mice

TABLE 1
COMPARATIVE DATA ON TITRATION OF EIGHT STRAINS OF POLIOMYELITIS VIRUSES IN SEVEN TISSUE CULTURE SYSTEMS

Strain of virus	Type	TDC50 obtained in several tissue culture systems							
		No serum		Serum present during cellular growth phase					
		Rh testis RT	Rh kidney LA RT	Rh kidney RT Parles + MS	Rh kidney 199 RT	Rh kidney 199 MI	HeLa ST	HeLa suspended cells	Human kidney
Brunhilde*	1	3 9	3 8	5 8	3 3?	—	<3 0	2 2	>6 0
Brooks	2	4 0	5 4	8 6	6 8	6 6	6 1	7 3	7 5
BEV-3	2	3 4	6 0	6 7	5 2	5 6	5 6	5 4	7 8
Fotovich	2	—	6 0	7 7	6 7	5 7	5 6	—	6 8
Linden	2	—	4 9	8 5	<7 0	5.3	5 6	6 2	<7 0
Orr	2	—	5 4	6 0	7 2	5 5	5 6	5 9	—
Y-SK	2	2 8	5 0	5 1	5 5	5 6	5 1	5 7	5 5
Saukett*	3	3 4	5 5	7 8	<5 0	—	—	—	5 5

RT = roller tube; LA = lactalbumin hydrolysate, MI = metabolic inhibition, ST = stationary tube, MS = monkey serum

* Pool prepared in Roux flasks in testicular implants, all others from monkey kidney implants

studied promptly lysed kidney cells, and replication of virus in high titer was achieved. Some of the data pertinent to this discussion appear in TABLE 1.

A comparison was made also of the reproducibility of virus titration in test systems involving monkey, human, and HeLa epithelial cell cultures These data also appear in TABLE 1 I am indebted to Doctor Jerome Syverton for the titration data obtained with HeLa cells and performed in his laboratory The results were quite uniform, and they indicate that preference of any one of the three systems must be one of choice, since corresponding end points were obtained with each of the eight strains of poliomyelitis viruses included in the test.

One other variable affecting measurements, particularly in titration of viruses, was the time of recording cell lysis TCD_{50} end points scored on the third day were almost always one or two logs lower than end points scored on the seventh day Time was of some, although comparatively less, importance in the behavior of most strains that had been passaged in tissue culture If the inoculum contained many infectious units (e g , 1000 TCD_{50}) replication was rapid, and maximal titers, based on growth-curve data, were often reached 24 to 36 hours after inoculation of actively growing cells After this time th[illegible] over a period of four days, indi-
[illegible]ay
[illegible]ite
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appear in TABLE 2

Comparative studies with established strains of viruses Another series of studies was made with reference pools of virus in order to compare the ID_{50} end point, using monkeys, mice, and kidney epithelial tissue cultures The reference pools consisted of monkey and of mouse CNS emulsions and fluids

TABLE 2

COMPARATIVE DATA ON FOUR STRAINS OF POLIOMYELITIS VIRUSES TITRATED AT INTERVALS IN HELA AND MONKEY KIDNEY EPITHELIAL CELL SYSTEMS

Time of harvest (hours)	In HeLa*		In monkey kidney†	
	Brunhilde	Brooks	Brunhilde	Brooks
Original	6.0	9.0	6.0	9.0
36	<6.0	5.0	6.3	7.5
60	8.5	7.0	5.8	7.0
84	7.5	—	5.6	6.5
108	6.5	6.5	5.0	5.7
132	6.5	—	5.1	5.0

* Virus grown in HeLa cells
† Virus grown in monkey kidney cells

TABLE 3

COMPARATIVE ID_{50} END POINT SCORES OBTAINED WITH REFERENCE POOLS OF POLIOMYELITIS VIRUSES IN MONKEYS, MICE, AND KIDNEY EPITHELIAL CELL CULTURES

Strain of virus	ID_{50} endpoint scores in								
	Monkeys			Mice			Tissue culture		
	Monkey CNS pool	Mouse CNS pool	TC* pool	Monkey CNS pool	Mouse CNS pool	TC pool	Monkey CNS pool	Mouse CNS pool	TC pool
Fotovich	5.7	6.1	7.0	0	5.2	3.8	6.5	8.2	8.0
Orr	5.8	>4.5	6.0	0	5.4	4.0	6.0	8.3	6.5
Lansing	Inc	4.0	4.5	—	5.4	4.2	3.7	<1.0	4.3
Y-SK	4.9	5.4	6.0	—	5.8	3.1	6.8	8.5	6.0

Inc = incomplete
Based on ID_{50}/gm or ml
* In trypsinized monkey kidney roller tubes

harvested from monkey kidney epithelial cells, all infected with several strains of poliomyelitis viruses. A summary of the results of these studies appears in TABLE 3.

The results may be considered both from observed and from certain theoretical aspects. The virus reference pools, namely those derived from monkey CNS and mouse CNS and from tissue cultures provided infectivity titers of corresponding values when each was tested intracerebrally in monkeys. Tissue culture virus gave somewhat higher end points, but the differences are not significant. When the virus pools were assayed in mice, ID_{50} end points obtained were numerically larger with mouse-adapted than with tissue culture-adapted virus. Assayed in monkey kidney epithelial cultures, the reference monkey CNS and tissue culture virus pools provided some irregular results. The data suggest that these two reference pools probably have equivalent end points. Mouse-adapted virus pools provided, with one exception, consistent and sig-

TABLE 6
COMPARATIVE TITRATIONS OF STOOL SPECIMENS IN TISSUE CULTURE

Source	Day of illness	Type	ID_{50} endpoints obtained in			
			HeLa ST*	Monkey kidney RT†	Monkey kidney MI†	Human kidney RT†
M Far	3rd	1	<1 7	2 0	1 7 or <	2 8
B Ke	7th	1	2 0	3 0	3 1	3 0
T L Bl	Healthy contact	3	1 0	4 5	4 2	3 3
R. Bl	13th	3	1 0	2 8	3 0	3 0

ST = stationary tube, MI = *metabolic inhibition*, RT = *roller tube*
* Titrations were done in the University of Minnesota, Minneapolis, Minn
† From same stool sample and the same dilution of stool, four tube test per dilution

TABLE 7
COMPARISON OF SEVERAL TISSUE CULTURE SYSTEMS
SERUM DILUTION END POINTS OF STANDARD ANTISERUM WITH PROTOTYPE VIRUSES

Virus*	Antiserum	Tissue culture system				Monkeys†	Mice†
		Testis R T	Kidney R T	HeLa S T	Color change kidney		
Brunhilde	Type 1	1,600	4,600	1,600	1,600	480	—
Lansing	Type 2	6,400	25,000	6,400	5,120	1,280	41,600
Saukett	*Type 3*	*6,400*	*18,000*	*6,400*	*6,144*	*4,500*	—

* With TCD_{50} 100 (range 50 to 200)
† Reciprocal of serum neutralization index

fecal extract An aliquot of the undiluted fecal extract was titrated separately in HeLa epithelial cell cultures

The results of the tests appear in TABLE 6 The data obtained are in agreement that, within the same experimental conditions, kidney epithelial cells, whether derived from monkeys or human beings, provide a more sensitive end point indicator of virus than HeLa epithelial cells The experimental evidence would suggest that the sensitivity ratio is 10 to 1,000:1

Comparison of tissue culture systems for antibody titrations The method has been established and its usefulness determined Any one of the methods discussed in this paper may be employed and corresponding end points obtained Comparative data on end points obtained employing standard high titer reference monkey serum with prototype viruses representing the three known serologic types of poliomyelitis viruses appear in TABLE 7. The figures given represent the mean values obtained in several tests

Summary

The studies reported here attest to the usefulness and to some of the experimental standards requisite to a systematic use of tissue culture methods in the study of poliomyelitis viruses The usefulness of tissue culture methods for a study of many biologic characteristics of poliomyelitis virus has been discussed

in this conference Its full application and the introduction of new approaches by its use augurs well for new and fundamental contributions The data presented here have been helpful to us in the assurance that recently isolated strains of poliomyelitis viruses can be assayed in tissue culture with a reliability equal to, if not better than, that achieved by older and more laborious methods

Strains of viruses adapted and passaged many times in extrahuman hosts (*e g*, animals, embryonated eggs, or tissue culture) undergo changes in their biologic character. It is in regard to these strains that comparative data in tissue culture and in experimental animals have provided, and will continue to provide, essential information necessary to a further understanding of these changes.

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COMPARATIVE SUSCEPTIBILITY OF TISSUE CULTURE CELLS IN EXPERIMENTAL ANIMALS AND MAN

By George A. Jervis

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With the development of variants (or mutants) of poliomyelitis virus under certain experimental conditions, it has become abundantly clear that some variants exhibit differences in host susceptibility.[1-7] The purpose of this presentation is limited to report pathological changes observed in tissue-culture cells, in monkeys, in rodents and, occasionally, in man, following inoculation with several variants of both type 1 and type 2 poliomyelitic virus

Methods and material The virus variants employed in the experiments have been developed in a manner previously reported[2-7] or described elsewhere in this monograph[8, 9] Inoculation in monkey kidney tissue cultures were made by conventional methods For inoculation in monkeys, the intracerebral, intraspinal and, occasionally, the intravenous routes were followed Rodents were inoculated by intracerebral and intraspinal routes In humans, [illegible]

obtained by a method essentially similar to that described by Bodian[11] For [illegible]

attempt was made to quantify the lesions in rodents In the human, some 20 blocks were cut at various levels of the spinal cord, and at least 25 sections were examined in each block

Results TABLE 1 shows the pathological changes observed with variants of the TN strain[2] The original strain produced severe lesions in the spinal cord of monkey by both the intracerebral and intravenous route In fact, in 28 animals, the cell loss in the anterior horn was over 75 per cent. The virus showed cytopathogenicity in tissue culture No pathological lesions were observed following inoculations in rodents The adaptation of the strain to cotton rats resulted in a variant which produced abundant lesions in rodents, but was considerably less pathogenic for the monkey than the original strain A mouse-adapted variant produced very mild pathological lesions in the monkey, two animals showing no cellular loss and 20 out of 22 animals less than 25 per cent cell loss In almost all animals in this group, there was no clinical evidence of motor deficit Cytopathogenic effect in tissue culture was obtained with some difficulty only after repeated passages This mouse-adapted variant, when fed to humans, caused no clinical manifestations of poliomyelitis Three individuals who were affected by incurable diseases died from one to two years after feeding of the virus No evidence of past poliomyelitis was found on pathologic examination of the spinal cord

TABLE 1

PATHOLOGIC LESION WITH TN STRAIN (TYPE 2)

Variants	Tissue culture*	Monkeys†			Mouse‡	Cotton rat‡	Human (orally)
		IC	IS	IV			
Original	+	28/28 (4)		2/0 (4)	0	0	?
Rat adapted	+	10/10 (2)	2/2 (2)		+	+	?
Mouse adapted	(+)	20/22 (1)			+	(+)	0/3
TC & cotton rat	(+)	14/56 (1)	1/1 (2)	1/4 (1)	(+)	+	?
TC & Swiss mouse	(+)	2/15 (1)		0/3	+		?
TC & PRI Mouse	0	1/24 (1)	2/21 (1)		+		0/12§

* Monkey kidney

† Figures in parentheses indicate the following (1) cell loss <25% (2) cell loss 26–50%, (3) cell loss 51–75%, and (4) cell loss >75%

‡ Both IC and IS

§ Clinically only

Another variant of TN virus obtained following numerous passages through tissue culture and adaptation to cotton rat caused clear-cut lesions in rodents but showed only mild pathogenicity for monkeys by the intracerebral route In fact, 42 out of 56 monkeys showed no lesions and, in 14, the total cell loss was less than 25 per cent. By the intraspinal route, pathogenicity was probably higher, but only two animals were studied Still milder lesions in the monkey were noted with a variant obtained through tissue cultures and adaptation to Swiss mice. Only two out of 15 animals showed lesions in the spinal cord The variant was apathogenic by intravenous injection Its effect by the intraspinal route was not investigated In tissue cultures, cytopathogenicity was obtained only after repeated passages The last variant listed in TABLE 1 resulted from tissue-culture passages and adaptation to PRI mice Pathogenicity for the monkey by both the intracerebral and intraspinal route was minimal, lesions in the mouse were clear cut, and no cytopathogenic effect was noted in tissue culture This variant was fed to 12 humans Clinically, no evidence of disease was noted

TABLE 2 shows the pathologic changes observed with a type 2 avianized strain [8] It may be seen that, in low chick embryo passages, the pathogenicity of the variant for monkey had decreased considerably as compared with the original strain while remaining unchanged for the mouse Cytopathogenicity in tissue culture was obtained only after repeated tissue passages Above the

TABLE 2

PATHOLOGIC LESIONS WITH MEF1 STRAIN TYPE 2

Variants	Tissue culture	Monkeys†			Mouse	Human (orally)
		IC	IS	IV		
Original	+*	12/12 (3)		1/1 (3)	+	?
Avian (6–40) pass	(+)	6/10 (2)			+	?
Avian (62–104) pass	0	5/28 (1)	2/11 (1)	0/8	+	0/2

TABLE 3

PATHOLOGIC LESIONS WITH SM STRAIN (TYPE 1)

Variants	Tissue culture	Monkeys†			Mouse‡	Cotton rat‡	Human (orally)
		I C	I S	I V			
Original	+	10/10 (4)		1/1 (4)	0	0	?
Rat adapted	+	11/11 (3)		1/1 (3)	+	+	?
Swiss mouse adapted	+	2/19 (1)		1/2 (1)	+	+	?
PRI mouse adapted	+	1/96 (1)	4/28 (1)		+	0	0/18§
PRI and human	+	0/74	2/20 (1)	1/1 (1)	+	0	0/2§

* Monkey kidney

† Figures in parentheses indicate the following (1) cell loss <25% (2) cell loss 26-50%, (3) cell loss 51-75% and (4) Cell loss >75%

‡ Both I C and I S

§ Clinically only

62 chick embryo passage, there was no cytopathogenic effect in tissue culture, and pathogenicity for monkey by all routes had decreased further, although mild lesions were still observed in about one fifth of the animals by either the intracerebral or the intraspinal route The lesions in rodents remained unchanged This last variant was fed to humans No clinical signs of disease were noted, and two individuals who died from malignant conditions several months after feeding showed no pathologic evidence of past poliomyelitis

TABLE 3 shows pathologic lesions obtained with variants of a type 1 strain The original SM strain[7] showed conspicuous pathogenicity for monkeys, 10 out of 10 animals showing severe pathologic lesions by the intracerebral route The strain was pathogenic for tissue culture and failed to produce lesions in rodents When adapted to cotton rats, the resulting variant produced lesions in rodents and exhibited cytopathogenic effect in tissue culture, while its pathogenicity for monkeys decreased somewhat as seen by the decrease percentage of cellular loss in the inoculated animals Adaptation of the strain to Swiss mice resulted in a variant which was pathogenic for rodents and tissue-culture cells, but much less pathogenic for monkey than the rat-adapted variant. Adaptation of the strain to PRI mice resulted in striking changes of susceptibility for monkeys, as demonstrated by the fact that only one out of 96 monkeys inoculated intracerebrally showed some mild pathologic lesions while, in the 95 remaining animals, no pathologic evidence of poliomyelitis was obtained On intraspinal inoculation, however, 4 out of 28 animals exhibited poliomyelitic lesions, the cell loss being low (less than 25 per cent) This variant was still pathogenic for mice and tissue cultures When fed to 18 human individuals, it caused no signs of disease No pathologic specimen was available in this group of humans The variant, when passed through the human intestinal canal, still preserved its characteristics of mildness, as seen by the observation that no monkey, out of 74, developed lesions following the intracerebral route and only 2, out of 20, by the intraspinal route

Comment The material here presented demonstrates remarkable differences in morphologic changes following inoculation of variants of poliomyelitic virus into various hosts, thus confirming from a pathologic point of view, data obtained mostly from clinical and immunologic studies It is interesting to note

that no decrease of pathogenicity for kidney cells was obtained in variants of type 1 while, with similar techniques, variants of type 2 were obtained which

monkeys by various routes. By the intravenous route, which was used sparingly, differences were noted among some variants in producing central nervous system lesions, whether because variants differ in their properties of systemic multiplication[10] or in their inherent ability to overcome the blood brain barrier, remains to be determined. By the intracerebral route, which was used extensively, variants producing no lesions were observed. The possibility that

ever, in animals in which the spread of the virus along the spinal cord was not sufficient to cause characteristic poliomyelitic lesions in regions removed from the point of inoculation, some difficulty was encountered in the interpretation of the pathologic changes. In fact, not infrequently, a traumatic myelitis with occasional secondary infection was encountered, accompanied by paralysis of the hind limbs, a clinico-pathologic picture which might be erroneously interpreted as caused by poliomyelitis virus.

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TABLE 3
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Variants	Tissue culture	Monkeys†			Mouse‡	Cotton rat‡	Human (orally)
		IC	IS	IV			
Original	+	10/10 (4)		1/1 (4)	0	0	?
Rat adapted	+	11/11 (3)		1/1 (3)	+	+	?
Swiss mouse adapted	+	2/19 (1)		1/2 (1)	+	+	?
PRI mouse adapted	+	1/96 (1)	4/28 (1)		+	0	0/18§
PRI and human	+	0/74	2/20 (1)	1/1 (1)	+	0	0/2§

(1) cell loss <25%, (2) cell loss 26–50% (3) cell loss 51–75%.

62 chick embryo passage, there was no cytopathogenic effect in tissue culture, and pathogenicity for monkey by all routes had decreased further, although mild lesions were still observed in about one fifth of the animals by either the intracerebral or the intraspinal route The lesions in rodents remained unchanged This last variant was fed to humans No clinical signs of disease were noted, and two individuals who died from malignant conditions several months after feeding showed no pathologic evidence of past poliomyelitis

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that no decrease of pathogenicity for kidney cells was obtained in variants of type 1 while, with similar techniques, variants of type 2 were obtained which were completely devoid of pathogenicity for kidney cells Pathogenicity for [illegible]

monkeys by various routes By the intravenous route, which was used sparingly, differences were noted among some variants in producing central nervous system lesions, whether because variants differ in their properties of systemic multiplication[10] or in their inherent ability to overcome the blood brain barrier, remains to be determined By the intracerebral route, which was used extensively, variants producing no lesions were observed The possibility that [illegible]

described by Li and Schaeffer[4] in rodents and, by Sabin,[6] in monkeys However, in animals in which the spread of the virus along the spinal cord was not sufficient to cause characteristic poliomyelitic lesions in regions removed from the point of inoculation, some difficulty was encountered in the interpretation of the pathologic changes In fact, not infrequently, a traumatic myelitis with occasional secondary infection was encountered, accompanied by paralysis of the hind limbs, a clinico-pathologic picture which might be erroneously interpreted as caused by poliomyelitis virus

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CELL SUSCEPTIBILITY TO VIRUSES

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To the outsider, the problem of cell susceptibility to poliomyelitis virus and, indeed, the whole of biology of poliomyelitis, appear as the geography of a wonderful country that has been visited mainly by hurried and harassed travelers, faced with immediate problems of survival. Recently, as the conference on which this monograph is based has proved, the need for real exploration has been recognized. At the same time, although probably not by coincidence, techniques have become available that will make this exploration possible Most of our knowledge of susceptibility to poliovirus, as illustrated by the preceding papers, has been gained from studies of susceptibility of vari- [illegible] the same organism or from [illegible] an outsider to attempt a [illegible] nstead, to a discussion of methodological and analogical approaches to the study of cell susceptibility in poliomyelitis

Poliovirus is a cytopathogenic virus, whose typical, although probably *not* exclusive interaction with host cells, is destructive. Latency or reservoir hosts have not yet been recognized, although work on some of the variant strains may well reveal some such situations Thus, the observational aspects of our problem are rather simple Cell susceptibility can be detected either by cell damage or by production of virus (or possibly, by production of some virus specific antigen). It is clear that both types of observation are required in any *study of cell susceptibility*, since *both noncytopathogenic and sterile*, nonreproducing virus variants appear to exist

The outcome of virus-host cell interaction will generally depend on three factors: the virus, the host cell, and the environment We shall discuss these factors one at a time

The Virus

The role of the virus is evident in the many reports of strains differing in cytopathogenicity and in affinity for various hosts and tissues. Differences in the type of cell damage evoked by virus may underlie the differences, reported elsewhere in this monograph by Dulbecco and Vogt, between plaque types produced by virus mutants in tissue cultures Strain differences may arise by various mechanisms, the models for which are provided by bacterial viruses

First, we have permanent, hereditary virus mutations Until recently, these appeared to be produced exclusively by intrinsic changes *in virus heredity* But the discovery of phage mutation occurring in irradiated host cells[1] indicates that virus mutations may result from still obscure interactions between the genomes of the virus and of the host Analysis of virus growth and mutation in cell suspensions may permit similar studies with polio virus

Second, we may have host-induced virus modifications[2] In phage, these

are observed as mass changes in the host-range of a virus, which are controlled by the last host cell where the virus has grown For example, a virus may have two hosts, A and B. Virus grown in host cells A may be able to infect and to grow in all cells A and all cells B, but virus grown on host B may multiply on all cells B and only on one out of 10^6 cells A. In phage, these changes are [illegible]

existence of such reversible adaptations

Clearly, the problem mentioned by Kibrick and others elsewhere in this monograph, as to whether the different titers of a virus preparation in different hosts reflect presence of mutants, continuous spectra of cell affinity, or variable but uniform probabilities of successful infection, is very relevant to the understanding of differences in host affinity Studies of the titers and the statistical distributions of infective units of virus passed back and forth between different host tissues, preferably in single growth cycles, should prove very fruitful

The Environment

Let us discuss next the role of the environment This is meant to include all factors external to the virus and to the host cell In virus infection of an animal, the environment includes the variety of organs, tissues, and circulating fluids that affect directly or indirectly, by mechanical or chemical means, the outcome of virus-cell interaction The importance of barriers is well appreciated by students of poliomyelitis More subtle environmental effects are revealed by studies such as those reported by Kaplan on monkey kidney, where differences are observed between the susceptibility of an organ and of cells from the same organ cultivated *in vitro*

In some situations, the influence of the internal environment on susceptibility may be trivial in mechanism as, *e g*, the role of high body temperature in preventing influenza infection of chickens[3] In other situations, humoral controls may affect cell or tissue susceptibility Of these, the serological ones are the best understood Hormonal controls, indicated by studies such as those reported here by Shwartzman and his co-workers, may be further elucidated by continued comparative studies of virus growth in tissue cultures, isolated organs, and intact animals

Another aspect of environmental effects is worth emphasizing, since it has probably not been appreciated sufficiently This concerns the profound effects that the artificial chemical environment may have on virus infection in tissue cultures[4] The medium may control adsorption, reproduction, and release of virus On the one hand, we may expect that a medium optimal for the growth of cultured cells may not be optimal for the growth of virus On the other hand, a medium may favor virus growth in one strain of host cells and may inhibit it in a different cell strain Just as nutritional and metabolic studies on bacteria have proved crucial to the study of bacterial viruses, studies on the nutrition and metabolism of pure lines of mammalian cells, such as those now

in progress in Eagle's laboratory at Bethesda, Md, will prove crucial for animal virology Here, of course, we are at the borderline between what we may legitimately consider as the role of the environment and what constitutes our next and last topic, the role of the host cell itself

The Host Cell

The role of the host cell is to be viewed in the light of our present conception of virus-cell interaction, as the performance of a joint task by an integrated functional system We do consider the host cell, not as a grazing ground, but as a factory in which the virus introduces an additional, often dominant blue print The suitability or unsuitability of the cell factory for the required task may depend on a number of causes, and may express itself in a variety of mechanisms

Causes of differences in susceptibility The differences in the aptitude of various cells to be host to a virus may be genetic, reflecting species, strain, and individual differences, or they may be developmental, as among various tissues and organs Any distinction between the two categories can hardly be more than descriptive so long as the metabolic and genetic events of differentiation remain one of the most obscure areas of biology Age differences in virus susceptibility may reflect developmental changes in cell susceptibility, as well as changes in internal environment Simpler cases for study may be provided by phenomena such as the different responses of chorionic and allantoic epithelia to influenza viruses[5] Even better material for study may be provided by *changes occurring in cultured cell strains A classic example* is that of a strain of rat fibroblasts that acquired susceptibility to eastern equine encephalomyelitis virus[6] The change in HeLa cell discussed here by Scherer may have been another example, but delays in analyzing this observation apparently robbed it of some of its potential interest

If I may extrapolate once more from the field of bacteriophage, I am inclined to predict that the study of the virus susceptiblity of closely related strains of cultured cells, in addition to its own significance for virology, may well do for cell differentiation what the study of susceptibility to phage did for bacterial variation, not only by providing tools for analysis, but also by stimulating interest and support for the study of developmental cell physiology.

Another cause of differences in cell susceptibility may be acquired *immunity* Although tissue-culture work has shown that the tissues of hyperimmunized animals are usually still sensitive to virus, interference phenomena, as well as earlier observations, provide examples of acquired cellular resistance. Although protection by interference is often of brief duration, probably lasting only until unprotected cells again become available, the possibility of more permanent cell protection by more or less latent virus infections is indicated by work on plant viruses and, especially, by work on lysogenic bacteria We know now that prophages, as permanent inhabitants of the chromosomes of lysogenic bacteria, not only confer immunity against reinfection by related phages, but can interfere specifically with the reproduction of unrelated ones Indeed, the lysogenic host cell may not only fail to support reproduction of the phage, but may even fail to be at all damaged by phages that would kill

related, nonlysogenic cells outright, by the very act of attachment (S. Lederberg, unpublished results) Thus, a latent virus can interfere with both the multiplication and the cytopathogenicity of completely unrelated viruses

Mechanisms of differences in susceptibility Differences in cell susceptibility may manifest themselves at any of the stages of virus-host interaction The stage may be the attachment of virus Kaplan's findings with susceptible and resistant kidney cells from various species of monkeys illustrate this possibility Differences in later stages of interaction may appear as differences either in virus yield or in the time required for virus production Extreme cases are those of sterile mutants, which produce abortive infections with or without cell destruction An abortive infection with cell destruction may lead to immunity by hypersensitivity, as in the cases of plants that localize viruses by a circumscribed necrotic response. With bacterial viruses, we recognize (or can artificially produce) abortive infections without any reproduction of viral material, abortive infections with failure to synthesize viral protein, and abortive infections with failure to assemble mature virus The observations of Dulbecco and Vogt on infection of single cells provide a recognizable relation between visible cell lesions and virus liberation This finding may make it possible to classify stages in the intracellular development of poliovirus and to decide at which stage an abortive infection is arrested Incidentally, chemotherapeutic programs may profit from a conscious search for agents that interfere selectively with specific stages of virus-host interaction

A final word on the possibility that abortive infection may be simulated by a special form of the host-induced modifications discussed earlier in this paper[2] With some phages, we find that a certain host bacterium Y produces, in place of normal virus, a full yield of a new virus type that is unable to grow in cells of strain X or of any of its relatives, but that still grows on other hosts, for example on Y. In Y cells, this virus again gives rise to perfectly normal virus, capable of growing on either X or Y In this case, mass infection of strain Y fails because of the host-imposed change on the virus, which represents, in a sense, a self-protection mechanism on the part of the host Phenomena of this kind might play a role in restricting virus reproduction and in determining the pathology and epidemiology of virus diseases

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PREVIOUS TONSILLECTOMY AND CURRENT PREGNANCY AS THEY AFFECT RISK OF POLIOMYELITIS ATTACK

By Ralph S. Paffenbarger, Jr., and Viktor O. Wilson
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Most humans acquire immunity to poliomyelitis through unapparent infections, while relatively few develop recognizable disease This directs interest to those factors of host and parasite that may convert an otherwise subclinical infection or abortive attack into nonparalytic or paralytic illness Major attention in this report is given to two independent host variables, previous tonsillectomy and current pregnancy, as they affect risk of poliomyelitis attack All data pertain to an outbreak in Olmsted County, Minnesota, 1952, when 215 cases occurred among 49,000 residents, an attack rate of 436 per 100,000 A striking number of adults was involved, one fourth of the cases being 20 years of age or older. Type 1 virus was the predominant if not the only virus type active, as evidenced by antibody determinations on serums of 98 child household contacts of cases More detailed epidemiologic and laboratory aspects are described elsewhere [1, 2]

Materials and Methods

Epidemiologic and clinical data on cases and their contacts were obtained during the progress of the outbreak, from July through November, 1952. Data on tonsillectomy, pregnancy, and other potential provoking factors were obtained in the summer and fall of 1954.

Observations recorded here are based on clinical cases examined by both physicians and physical therapists and reported to health authorities Both paralytic and nonparalytic cases are considered, the latter comprising one fifth of total cases

Definition of terms Paralytic poliomyelitis was defined as an acute febrile illness with signs of central nervous system involvement and demonstrated loss of muscle power due to paralysis, apart from muscle stiffness Bulbar cases

poliomyelitis was defined as an acute febrile illness with central nervous system signs including muscle stiffness and abnormal cerebrospinal fluid findings detected during the acute or convalescent stage of illness (nonbulbar poliomyelitis refers to patients with either spinal or nonparalytic disease) These definitions consider muscle evaluations performed between the sixth and ninth month after onset of illness as distinct from definitions in prior reports on this outbreak,[1, 2] which dealt only with hospital discharge diagnoses. This change in

definitions has shifted 8 of 54 cases previously diagnosed as nonparalytic into paralytic categories

In studies reported elsewhere,[2] child household contacts of recognized paralytic and nonparalytic cases were bled as soon as possible after the index case occurred and, one month later, to determine frequency of viremia and specific antibody response Histories of illness were obtained that covered the period of one month before, to one month after, the date of first bleeding [illegible]

as subclinical poliomyelitis infections

Contact is defined as personal association between a poliomyelitis case and another individual *in a home or on home premises at any time in the week* surrounding the date of onset of the case Attack rates of poliomyelitis were twice as high in individuals with such contact ("contacts") as in the remainder of the population[1]

Tonsillectomy refers to surgical removal of palatine (faucial) tonsils with or without excision of adenoids No poliomyelitis case or associate underwent tonsillectomy within two months prior to illness or contact

Differences in groups being compared, as measured by statistical analyses, are considered significant at a probability level of 05

Contact studies Prospective studies in 1952 had shown that 3,752 individuals were in contact with a poliomyelitis case under the criteria defined Fifty of these contacts subsequently developed the disease Contacts without recognizable poliomyelitis represent a favorable group for comparison with the 215 cases in the outbreak since they were at great risk of infection[2] Thus frequencies of tonsillectomy, pregnancy, and other factors were determined, in 1954, as they pertained to case and contact groups in 1952 Return mail questionnaires were sent to the head of households in case and contact groups after current home addresses were established After five months, and after second and third questionnaires in some instances, information was received for all cases and nearly 85 per cent of contacts Through telephone calls and home visits, data were obtained for all other contacts still living in the Olmsted County area All told, adequate information was received for 3,297 (89 per cent) of the 3,702 contacts without poliomyelitis No significant difference in frequency of tonsillectomy or pregnancy existed between those who responded to original mail questionnaires, follow-up letters, telephone calls, or home visits This fact supports the assumption that the 405 contacts who could not be reached did not differ from the remainder These 405 were excluded from the study

Survey studies Frequency of tonsillectomy in the population-at-large was estimated, in 1954, by questioning 588 individuals randomly selected from an Olmsted County directory that contained names and addresses of all adult county residents Telephone calls and home visits provided data on individuals in the random sample and members of their families Information usually was given

Table 2

Comparison of the Frequency of Prior Tonsillectomy Among Poliomyelitis Cases, Noncase Contacts, and a Survey Population, by Occupation of Head of Household

Occupational groups	Poliomyelitis cases			Contacts without poliomyelitis			Survey population		
	Number	With tonsillectomy		Number	With tonsillectomy		Number	With tonsillectomy	
		Number	Observed per cent		Number	Adjusted per cent*		Number	Adjusted per cent*
Professional and managerial	59	28	47	712	319	39	488	240	36
Clerical and skilled	55	15	27	993	316	24	667	257	25
Semiskilled and unskilled	52	12	23	813	198	14	476	122	14
Farm	49	11	22	779	191	14	553	123	15

* Age adjusted to number of poliomyelitis cases, by occupational groups

Table 3

Frequencies of Prior Tonsillectomy Among the Gradient of Poliomyelitis Cases, Noncase Contacts, and a Survey Population

Categories	*Individuals*			
	Total number	With tonsillectomy		
		Number	Per cent	Adjusted per cent*
Poliomyelitis cases				
Bulbar (or bulbospinal)	66	32	48	
Spinal alone	103	24	23	27
Nonparalytic	46	10	22	28
Contacts without poliomyelitis	3,297	1,024	31	25
Survey population	2,184	742	34	25

* Age adjusted to number of bulbar poliomyelitis cases

myelitis associated with prior tonsillectomy was focused to an excess of bulbar involvement and its rather frequent consequence of fatal outcome They also suggest that tonsillectomy, or those factors and clinical conditions which incite it, tended to convert unrecognizable and nonbulbar poliomyelitis into

of county residents who had and who had not undergone this operation,

TABLE 4

FREQUENCIES OF PRIOR TONSILLECTOMY AMONG THE GRADIENT OF POLIOMYELITIS CASES, NONCASE CONTACTS, AND A SURVEY POPULATION, AGED 0 TO 14 YEARS

Categories	Individuals aged 0 to 14 years			
	Total number	With tonsillectomy		
		Number	Per cent	Adjusted per cent†
Poliomyelitis cases				
Bulbar (or bulbospinal)	42	18	43	
Spinal alone	70	6	9	13
Nonparalytic	35	4	11	14
Presumed abortive cases*	51	4	8	10
Presumed subclinical cases*	41	3	7	11
Contacts without poliomyelitis	1,389	204	15	16
Survey population	658	96	15	15

* Defined in text
† Age adjusted to number of bulbar cases

more striking In the tonsillectomized population, the attack rate of bulbar disease was 189 per 100,000 and the mortality rate 41 per 100,000 Corresponding rates in the nontonsillectomized population were 63 and 24

In other words, there were 32 bulbar cases, 7 of whom died, in the population of Olmsted County that were estimated to have experienced tonsillectomy This contrasts with the expected number of 11 bulbar cases, with 4 fatalities, if rates in the nontonsillectomized populace pertained So the tonsillectomized population contributed the excess 21 cases, with 3 deaths

Tonsillectomy and bulbar poliomyelitis In a comprehensive report on 2,699 cases from the state of Minnesota, 1946, Anderson and Rondeau[14] have shown association between prior tonsillectomy and predisposition to the bulbar form in individuals developing recognizable poliomyelitis This association persisted when correction was made for age, sex, and the average number of persons per room in the place of residence (The latter factor measures economic and related standards)

In the Olmsted County outbreak, half again as many bulbar (48 per cent) as nonbulbar cases (27 per cent) had undergone tonsillectomy (TABLE 3) These data represent a much smaller experience than that of Anderson and Rondeau, but offer opportunity to examine such factors capable of biasing obobservations as (1) changing virulence of virus, and (2) human economic and cultural patterns

For instance, a rather prominent shift from younger to older cases was noted as the outbreak progressed[1] Since adults are more apt to have experienced tonsillectomy than children, any chronological increase in virulence of the infectious agent would increase the proportion of bulbar and fatal cases at a time when adults were developing the disease The predisposing cause of bulbar disease, in such circumstance, would be enhanced virulence of virus rather than tonsillectomy Data in TABLE 5 bear on virulence as measured by clinical forms of poliomyelitis in four successive periods corresponding to intervals when

TABLE 5

CHRONOLOGICAL DISTRIBUTION OF POLIOMYELITIS CASES INTO CASE-QUARTILES* BY CLINICAL FORMS OF DISEASE

Case-quartiles 1952	Type disease	Poliomyelitis cases					
		Age in years			Totals		
		0–4	5–14	15+	No	Per cent	Expected per cent†
1 July 4–31	Nonparalytic	4	3	0	7	13	22
	Spinal alone	17	10	4	31	57	50
	Bulbar and fatal	5	5	6	16	30	28
	Totals	26	18	10	54		
2 Aug 1–16	Nonparalytic	3	7	2	12	22	22
	Spinal alone	8	8	5	21	38	47
	Bulbar and fatal	5	12	5	22	40	31
	Totals	16	27	12	55		
3 Aug 17–Sept 10	Nonparalytic	5	7	3	15	28	21
	Spinal alone	11	5	11	27	51	49
	Bulbar and fatal	0	4	7	11	21	30
	Totals	16	16	21	53		
4 Sept 11–Nov 26	Nonparalytic	2	4	6	12	23	21
	Spinal alone	3	8	13	24	45	46
	Bulbar and fatal	4	7	6	17	32	33
	Totals	9	19	25	53		

* Total cases apportioned into sequential quarters according to dates of onset
† Expected per cent when frequencies of clinical types of poliomyelitis are made specific for age distributions in the total outbreak

equal numbers of cases had onset of disease (case-quartiles). Results are similar when cases are distributed by dates of onset in equal quarters of time (time-quartiles)

Considerable variation existed by specific age classes among case-quartiles For example, a larger proportion of bulbar cases aged 5 to 14 years developed in the second quartile than in prior or subsequent periods Also, decreasing proportions of bulbar cases over 14 years of age occurred in sequential quartiles The indirect effect of the latter is to reduce the difference in frequency of tonsillectomy between bulbar and nonbulbar cases. In general, however, there was no consistent chronological trend in clinical forms of poliomyelitis Too, no differences were observed in percentages of cases, specific for age and case-quartiles, having residual muscle weakness six to nine months after onset of illness Thus, these data tend to dispel the possibilities that (1) virulence of virus increased with progress of the outbreak, and (2) bulbar disease was re- ... lationship

10-

TABLE 6

PROPORTIONS OF BULBAR AND NONBULBAR POLIOMYELITIS CASES WITH HISTORY OF PRIOR TONSILLECTOMY, BY OCCUPATION OF HEAD OF HOUSEHOLD

Occupational groups	Bulbar cases				Nonbulbar cases			
		With tonsillectomy				With tonsillectomy		
	Number	Number	Per cent	Expected No.*	Number	Number	Per cent	Adjusted per cent†
Professional and managerial	17	11	65	8.0	42	17	40	47
Clerical and skilled	13	6	46	5.2	42	9	21	40
Semiskilled and unskilled	21	9	43	1.4	31	3	10	7
Farm	15	6	40	2.8	34	5	15	19

* Expected number if frequency of tonsillectomy in nonbulbar cases had prevailed by specific age and occupational groups

† Age adjusted to number of bulbar cases

myelitis, the association between tonsillectomy and bulbar disease is supported by taking account of cultural and related factors. This is done in TABLE 6, which contrasts, by occupational groups, proportions of bulbar cases with his-

Other provoking factors. Intramuscular inoculation of antigens, well established as predisposing to localization of paralysis when given within the month prior to onset,[17, 21] and less well established as increasing incidence[22] can be eliminated as a potential bias in this study. Only three patients received immunizing antigens in the month before poliomyelitis (two without history of tonsillectomy), so this factor could have had little influence. Moreover, routine immunization procedures were discontinued by local physicians during the entire period of epidemicity. Also, patients known to have received penicillin parenterally in the initial days of illness, were distributed equally between tonsillectomized and nontonsillectomized groups in proportions of 9 and 8 per cent.

As will be shown later, similar proportions of pregnant and age-comparable nonpregnant cases had experienced previous tonsillectomy, so the provoking effect of pregnancy does not influence these data.

Pregnancy and Poliomyelitis

Using [illegible] available, Anderson

that influence attack, taking due notice of the mechanism of transmission, namely personal contact with cases and with children who were especially prone to spread the disease.[1, 2]

TABLE 7 shows that six (7 per cent) of 89 pregnant women who were per-

TABLE 7

COMPARISON OF ATTACK RATES IN PREGNANT AND NONPREGNANT FEMALE CONTACTS OF POLIOMYELITIS CASES

Age (years)	Pregnant contacts				Nonpregnant female contacts		
	Number	Poliomyelitis cases	Attack rate per cent	Expected cases*	Number	Poliomyelitis cases	Attack rate per cent
15–24	27	1	4	0 5	176	3	2
25–34	50	4	8	1 0	290	4	1
35–44	12	1	8	0	185	0	0
Totals	89	6	7	1 5	651	7	1
Age-adjusted attack rate $\frac{(1\ 5)}{(89)}$ *in per cent†*							1

* Expected cases if attack rates in nonpregnant female contacts had prevailed
† Adjusted to number of pregnant contacts

sonally associated with previous cases of poliomyelitis, as defined, developed the disease This compares with seven (1 per cent) of 651 nonpregnant females in childbearing age classes who were in similar contact with a case Age-adjustment does not alter the difference

Equal proportions of women (75 per cent) in each population came from households with one or more children under 15 years of age, and average family sizes were similar The importance of the latter observation lies in the fact that children were chiefly responsible for the introduction of poliomyelitic infection and disease into households[1,2] Since such comparability reigns between populations of pregnant and nonpregnant contacts of cases, it is logical to conclude that the difference in attack between these populations indicates direct association between pregnancy and incidence of poliomyelitis

Estimates from decennial census figures and from birth records for Olmsted County provided data on pregnancy and marital status for residents in childbearing age classes (15 to 44 years) These estimates, which showed an excess of married women over married men that balanced out only in age classes beyond 44 years, permit mea poliomyelitis TABLE 8 show who were pregnant at some an attack rate of 0 9 per cent married females and 19 of 5,700 married males contracted poliomyelitis, age-adjusted rates of 0 4 per cent Further, six nonmarried female and the same number of nonmarried male cases occurred in their respective populations, age-adjusted rates of 0 1 and 0 2 per cent These data agree with findings in the smaller number of female contacts of cases

Other differentiating factors must be considered before accepting the in-

tack Comparability of groups under study is suggested in TABLE 9, which

TABLE 8
COMPARISON OF POLIOMYELITIS ATTACK RATES IN MARRIED FEMALES OF CHILDBEARING AGES AND AGE COMPARABLE MARRIED MALES

Age (years)	Married females						Married males		
	Pregnant			Nonpregnant					
	Population	Cases	Attack rate per cent	Population	Cases	Attack rate per cent	Population	Cases	Attack rate per cent
15–24	695	9	1.3	897	2	0.2	495	1	0.2
25–34	1,076	8	0.7	2,203	14	0.6	2,710	13	0.5
35–44	255	1	0.4	2,640	1	<0.1	2,527	5	0.2
Totals	2,026	18	0.9	5,740	17	0.3	5,732	19	0.3
Age adjusted attack rate per cent *						0.4			0.4

* Adjusted to pregnant population

TABLE 9
COMPARISON OF VARIOUS ATTRIBUTES IN MARRIED FEMALE AND MARRIED MALE POLIOMYELITIS CASES AGED 15 TO 44 YEARS

Attributes	Married female cases				Married male cases	
	Pregnant		Nonpregnant			
	Number	Per cent of total	Number	Per cent of total	Number	Per cent of total
Total cases	18		17		19	
Type disease						
Nonparalytic	3		4		2	
Spinal alone	12		6		10	
Bulbar or bulbospinal	2	83	3	76	5	89
Fatal (bulbar)	1		4		2	
Residual weakness at 6 to 9 months	10 13	77	6 8	75	13 15	87
Contact*	6	33	4	24	6	32
Children in household	14	78	13	76	17	89
Average family size	3.6		3.9		3.9	
Prior tonsillectomy	10	56	10	59	9	47

* Defined in text

shows no significant differences in the various attributes tested. Roughly one third of the cases in each group reported contact, as defined, with a prior case. Three fourths had children less than 15 years of age living in their household. Half had undergone prior tonsillectomy. Three fourths were classified as paralytic with residual weakness between the sixth and ninth month after onset. Average family sizes were similar. Also, these attributes appeared in the six nonmarried female and six nonmarried male cases in similar proportions to those in pregnant cases. This absence of biasing factors between pregnant cases and other groups obviates need for adjustment and strengthens the thesis that pregnancy increases susceptibility to poliomyelitis attack.

The significance of the provoking effect of pregnancy in this outbreak needs clarification. Eighteen poliomyelitis cases developed in pregnant women as compared to an expected number of 8 cases if age-specific attack rates in nonpregnant married women had pertained. Thus, approximately 10 cases can be attributed to the gravid state.

Hunter and Milliken[24] compared clinical forms of poliomyelitis between 49 pregnant cases, including the 18 reported here, and 141 nonpregnant female cases who were admitted to St Mary's Hospital, Rochester, Minn, from 1944 through 1952 Their data revealed similar case fatality rates and similar frequencies of bulbospinal disease in pregnant and nonpregnant cases But women who contracted poliomyelitis in the second and third trimester of pregnancy were more apt to have the bulbospinal form and to die than women who became ill during the first trimester.

Discussion

Observations reported here reveal an association between previous tonsillectomy and an increased risk of poliomyelitis attack that results in an excess [illegible] however, [illegible] s or con- [illegible] provoking elements Yet, the constancy of the association among age and occupational groups that experienced different frequencies of tonsillectomy, together with the impression that little constitutional difference exists between tonsil-present and tonsil-removed individuals, leads us to believe that susceptibility to poliomyelitis is enhanced by tonsillectomy *per se*. Studies are needed on a larger series of cases, (1) to determine indications for tonsillectomy; (2) to make careful note of the timing of bulbar symptoms relative to other sites of paralysis (*i e* bulbospinal versus spinobulbar), and (3) to treat pure bulbar cases as a distinct group Perhaps ultimate conclusion awaits experimental study in laboratory primates

A long-term provoking effect of tonsillectomy urges speculation on the pathogenetic mechanism involved Two hypotheses are intriguing—direct entry and spread of virus to the medulla via nerves supplying the pharynx and palate, versus increased penetrability of capillaries to circulating virus in the region of the brain stem supplying innervation to tonsillar tissues. Recent experiments by Bodian[25] provide data supporting the latter hypothesis as the explanation of the provocative effect of inoculations and trauma His findings make functional vascular changes especially appealing as an interpretation of the effect of tonsillectomy

Increased risk of poliomyelitis during pregnancy presumably relates to endocrinologic changes that alter defense barriers at sites of virus multiplication or invasion Here again, laboratory studies may offer definitive evidence on mechanisms involved

Three other points need emphasis (1) estimates indicate that the influence of tonsillectomy and pregnancy in this outbreak contributed approximately 31 (14 per cent) of the 215 cases, including three deaths, (2) findings reported here stress the need for firm indications before tonsillectomy is undertaken, (3) preg-

nant women deserve consideration for immunization against poliomyelitis when a suitable vaccine becomes available

Summary

This report describes previous tonsillectomy and current pregnancy, two independent host variables, as they affect risk of poliomyelitis attack Data pertain to an outbreak in Olmsted County, Minnesota, 1952, which was largely if not solely due to type 1 virus It was established that:

(1) Thirty-one per cent of 215 cases had undergone tonsillectomy more than two months prior to the outbreak, as contrasted to age-adjusted frequencies of 23 per cent in 3,300 contacts of cases, and 22 per cent in 2,200 randomly selected [illegible] attack

(2) Forty-eight per cent of 66 bulbar cases had undergone prior tonsillectomy as opposed to an age-adjusted frequency of 27 per cent of 149 nonbulbar cases This significant difference persists when account is taken of such potential variables as economic and cultural factors of host, and changing virulence of parasite Thus the increased incidence of poliomyelitis associated with prior tonsillectomy was limited to an excess of cases with brain stem involvement and its frequent consequence of death

(3) Seven per cent of 89 pregnant contacts of cases developed poliomyelitis as compared to 1 per cent of 651 nonpregnant female contacts of childbearing ages Also, the attack rate among all pregnant females in the county was significantly higher than rates in nonpregnant females and in males of similar ages All groups seemed comparable as to other factors which influence incidence These findings offer presumptive evidence that susceptibility to clinical poliomyelitis is enhanced by pregnancy

(4) Estimates indicate that tonsillectomy, or those conditions which prompt this operation, was responsible for an estimated 21 (10 per cent) of the 215 cases, including three deaths Pregnancy contributed approximately 10 of the remaining cases

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ENDOCRINOLOGICAL ASPECTS OF PATHOGENESIS OF EXPERIMENTAL POLIOMYELITIS*

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Susceptibility to poliomyelitis, whether studied in laboratory animals or observed in the human population, cannot be measured solely by immunologic barriers It is apparent that additional factors are operative in determining predisposition to the spontaneous disease Somatic stress and pregnancy have been equated with decreased resistance to poliomyelitis by numerous observers Experimentally, gross alteration in susceptibility to the inoculated disease is immediately apparent following cortisone administration in all inherently susceptible species [1, 2]

In the initial experiments, the Syrian hamster was chosen because of its partial native refractoriness to the disease, thus allowing an opportunity to observe both increase and decrease in paralytic incidence consequent to experimentally introduced factors The cortisone-potentiated disease in the hamster afforded a model to study the pathogenesis of experimental poliomyelitis serving also to clarify the progression of the virus in its course from the periphery to the critical tissues—the central nervous system

Two bodies of information thus became available First, a pathway traveled by the virus and, specifically, the extraneural preparalytic sites of viral propagation [3, 4] Second, the observation that a wide range of endocrine disturbances were capable of materially altering susceptibility to the introduced pathogen in experimental animals [5] It was necessary to determine whether there was any basic pattern in the diverse endocrinologic procedures instituted, whether such procedures had any discriminate action upon the specific tissues amenable to poliomyelitis virus proliferation, and, finally, whether an understandable correlation existed between the two

Pathogenesis of Peripherally Inoculated Poliomyelitis Virus in the Syrian Hamster

The Syrian hamster is totally refractory to parenterally introduced poliomyelitis virus Concomitant cortisone therapy however, allows the development of a fulminant and usually lethal paralytic infection following intraperitoneal, intramuscular, or subcutaneous administration of the virus During the course of this augmented infection, microscopic lesions are noted in the striated musculature[6] and, within a form of specialized adipose tissue, are referred to as brown fat [7, 8] This latter tissue, while detectable in all mammalian species, is more abundant in members of the rodent family In contrast to the unconfined nature of white fat, brown fat generally displays a defined pattern of anatomic distribution It is found in the axillary cavity, between the scapulae, encompassing the adrenal glands in intimate relationship to the

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc., New York, N. Y.

TABLE 1

IRUS PROGRESSION IN CORTISONE-TREATED HAMSTERS FOLLOWING VARIOUS ROUTES OI INOCULATION EXPRESSED IN —LOGS LD_{50} IN MICE

Virus route	Duration of infection in hamsters	Vira concentration in tissues			
		Interscapular brown fat	Periadrenal brown fat	Cord	Brain
ntraperitoneal	24 hrs	—	2.0	0	—
	48 hrs	—	3.78	0	—
	72 hrs	4.78	5 0	0	—
	96 hrs	—	5.0	3 4	—
	120 hrs	—	4 7	3.78	—
ntramuscular front left leg	18 hrs	3.3	0	0	—
	48 hrs.	4 0	0	0	—
	72 hrs	4 9	2.6	3 3	—
	96 hrs	4.9	2.7	3 78	—
ubcutaneous front left leg	18 hrs	1.7	—	—	—
	72 hrs	4 6	—	—	—
	96 hrs	5.7	—	—	—
	120 hrs	5 0	—	—	—
ntracerebral	24 hrs	2 7	—	2 0	3 7
	48 hrs	4 3	—	3 0	4 1
	96 hrs	5 78	—	3 48	3 78
	120 hrs	5 3	—	3.7	3 7

Minus (—) = not tested

ymus, and running parallel to the vertebral column bilaterally. In contra istinction to white fat, it tends to be lobulated and grossly discrete.

Large numbers of male Syrian hamsters, generally between 20 to 30 gm ir ody weight, were inoculated by various parenteral routes with type 2 (MEF rain) poliomyelitis virus A course of cortisone therapy was also given nimals from various groups were sacrificed, generally at 24-hour intervals nd immediately autopsied Selected tissues were harvested aseptically, and heir viral content was titrated by means of intracerebral mouse inoculation.

As may be seen from TABLE 1, little virus could be detected anywhere in the amster body 24 hours after intraperitoneal inoculation. The small amount ound in the periadrenal brown fat may have possibly represented an infective aterial residue initially introduced In the succeeding 48 hours, there was a ery rapid proliferation of the virus within the periadrenal brown fat reaching vels of -5 logs LD_{50}. Quantitation of virus in the brown fat elsewhere in e body disclosed an equivalent amount of virus proliferation. Thus, within e first 96 hours following intraperitoneal inoculation of the virus into hamers, the principal sites of viral activity found were within the various visceral ci of brown fat Some viral proliferation, in addition, occurred within the aravertebral skeletal musculature No virus, however, could be detected ithin the brain or spinal cord at this time Animals sacrificed at 96 hours, nd beyond, generally showed a perceptible amount of virus within emulsions repared from spinal cords At no time did the titer of virus within the central ervous system exceed or even equal that found within the extraneural sites of rown fat The appearance of the virus within the spinal cord coincided with

the clinical presentation of paralysis and with the classical anterior horn cell inflammatory changes.

When the virus was introduced intramuscularly into the upper limb of cortisone-treated hamsters, an essentially similar pattern of pathogenesis emerged (TABLE 1). Significant quantities of virus could be isolated from the intrascapular brown fat within 18 hours following introduction of the virus into the

virus content in

t belatedly, the

within 72 hours

after the time of initial virus introduction. Detectable amounts of virus within the spinal cord were not found prior to 72 hours. Again, as in the case of animals studied following intraperitoneal introduction of the virus, the ultimate level of virus content within the spinal cord never achieved that observed within the brown fat. The striated muscle at the immediate site of virus introduction also contained large quantities of virus.

Subcutaneous inoculation of the virus in cortisone-treated hamsters also resulted in a considerable virus proliferation within the intrascapular brown fat (TABLE 1).

When the virus was introduced directly into the brain of cortisone-treated hamsters, measurable amounts of virus could be demonstrated within the interscapular brown fat as early as 24 hours following the intracerebral inoculation. Even by this route, the quantitative extent of virus propagation within the brown fat was greater than within the brain or spinal cord. At 96 hours, there was at least a 2-log increase in virus content within brown fat, as compared to either the brain or spinal cord.

Approximately 48 hours after intraperitoneal introduction of virus into cortisone-treated hamsters, a viremia occurred which persisted about three days (TABLE 2).

Histopathology of Brown Fat Necrosis Caused by the MEF$_1$ Strain of Poliomyelitis in Cortisone-Treated Hamsters

Parallel studies of the microscopic alterations within the brown fat were carried out at various intervals of infection, and following various routes of virus administration. Viral propagation within brown fat was invariably accompanied by a well-defined series of histologic changes within this tissue. The

TABLE 2
VIREMIA FOLLOWING INTRAPERITONEAL INOCULATION OF MEF$_1$ INTO CORTISONE TREATED HAMSTERS

Duration of infection prior to bleeding	Viral concentration in blood of individual hamsters logs LD_{50} in mice		
18 hrs	0	0	0	0
45 hrs	2.88	3	3	3.3
72 hrs	2.7	1	3.78	3.3
96 hrs	0	2.88	3	3.4
120 hrs	0	0	1.7	3

TABLE 3

CYNOMOLGUS MONKEYS INOCULATED INTRAMUSCULARLY WITH WISCONSIN STRAIN POLIOMYELITIS VIRUS

	Untreated	Cortisone treated
Total number of animals	12	12
Days of incubation		
Range	4-15	5-24
Median	7	7
No with CNS lesions	12	12
No with brown fat lesions	11	12
Virus in cervical cord, median $-\log TCD_{50}$	4 5	5 0
Virus in brown fat, median $-\log TCD_{50}$	5 0	4 0

initial reactions were of an intracellular variety. The brown fat cell cytoplasm, normally composed of delicate interlacing filaments, assumed a coarse smudginess Very rapidly thereafter, a granular necrosis of the cytoplasm ensued, involving, somewhat belatedly, the nucleus The resultant detritus retracted peripherally, leaving a central zone cleared of either structure or debris The compact centripetally situated residual tissue rapidly became basophilic The supraimposed coloration was caused by a calcific deposition and was demonstrable within 24 hours after the onset of cellular degradation Only during the final stage of brown fat necrobiosis was an inflammatory response manifest

Pathogenesis of Experimental Simian Poliomyelitis

Following parenteral introduction of the poliomyelitis virus type 1 (strains Wisconsin, MacMahon, Brabyn, Brunhilde) or type 3 (strains Farrabaugh, Saukett), histologic lesions and high levels of virus were detected within the axillary brown fat as early as the third day following inoculation. As will be seen from TABLE 3, cortisone treatment was not necessary, as in the case of hamsters, for the production of microscopic lesions within brown fat or for the propagation of virus within such tissue The lesions within monkey brown fat, while not as extensive as those described above in hamster brown fat, were of essentially similar pathologic character [9]

In cynomolgus monkeys sacrificed 72 hours after intramuscular injection of the Wisconsin strain of poliomyelitis virus, no virus could be isolated from the *spinal cord and no histologic lesions characteristic of poliomyelitis* were demonstrated Nevertheless, virus could be isolated from the blood, as well as from [illegible] regions (TABLE 4)

crificed

, well as

the presence of very early ganglionic lesions within the *anterior horns* of the spinal cord.

The myositis that characterized the extraneural progression of the poliomyelitis virus in the hamster was never demonstrable in the infected and paralyzed cynomolgus monkey (TABLE 5)

TABLE 4

VIRAL CONCENTRATION FOLLOWING INOCULATION INTO CYNOMOLGUS MONKEYS EXPRESSED IN −LOG TCD50

Route of inoculation of Wisconsin strain	Intravenous		Intramuscular (left arm)	
Hours after inoculation	72 hrs	96 hrs	72 hrs	96 hrs
Blood	5 0	3 0	3 0	4 0
Left axillary brown fat	3 5	6 3	5 6	7 0
Right axillary brown fat	3 3	6 0	6 3	6 3
Spinal cord	2 5	3 0	0	3 5

TABLE 5

VISCERAL LESIONS FOLLOWING PERIPHERAL INOCULATION OF POLIOMYELITIS VIRUS

Host	Golden hamster		Cynomolgus monkey	
Viruses	MEF1 Lansing Yale SK		Wisconsin Brabyn (Ward) McMahon Brunhilde Farabaugh Saukett	
	Untreated	Cortisone treated	Untreated	Cortisone treated
Peripheral takes	−	+	+	+
Brown fat lesions	−	+	+	+
Muscle lesions	−	+	−	−

Relationship of Spontaneous or Induced Endocrine Disturbances to Poliomyelitis Susceptibility in the Syrian Hamster

Extended studies indicated that hamsters subjected to the normal yearly climatic variations responded with fluctuation in adrenal, thymic, testicular, and brown-fat weight Significant deviation in mortality and morbidity rates was also noted following intracerebral inoculation of poliomyelitis virus (strain MEF1) in the Syrian hamster, when such procedure was carried out at different times of the year[4] It became apparent that there was some statistical relationship between adrenal size, as expressed in mg per 100 gm of body weight, and susceptibility incident to the inoculated disease (TABLE 6) Thus, during the early spring of 1953, when the average adrenal weight was 17 00 mg. per 100 gm of body weight, the morbidity was 66 9 per cent In the succeeding period (late spring and early summer), there was noted a progressive diminution in adrenal weight, there being 13 51 mg of adrenal tissue per 100 grams of body weight during this period Concomitantly, the morbidity rate dropped to 43 5 per cent during this period In early fall of the year under study, adrenal weight in a large number of animals analyzed for this purpose to a level equivalent to that noted during the early spring In parallel the

TABLE 6
RELATION OF SEASONAL VARIATION IN WEIGHT OF ADRENAL GLAND TO SEASONAL FLUCTUATION IN SUSCEPTIBILITY TO POLIOMYELITIS IN THE SYRIAN HAMSTER

Period	Variation in adrenal wgt		Variation in susceptibility		
	No animals	Mean wgt. mgm /100 gm body wgt	No animals	Morbidity	Mortality
4/1-5/15	60	17 09	139	66 9%	38 8%
5/22-7/10	144	13.51	85	43 5%	23 5%
9/1-10/30	179	17 17	218	65 1%	36 7%

TABLE 7
RELATION OF TESTICULAR MATURITY TO POLIOMYELITIS SUSCEPTIBILITY IN THE SYRIAN HAMSTER

Body wgt.	Testicular activity*			Variation in susceptibility		
	No animals	Immature %	Highly active %	No animals	Morbidity %	Mortality %
15-25 gm	37	97	0	58	81 03	60 34
25-55 gm	232	36	9	238	62.18	32 35
55-70 gm	26	35	61	58	63 79	27 58

* Testicular activity determined by relative gonadal weight Testes weighing less than 300 mgm /100 gm body weight were considered immature Testes weighing over 600 mgm /100 gm body weight were deemed highly active

TABLE 8
EFFECT OF INDUCED ENDOCRINE DISTURBANCES UPON SUSCEPTIBILITY OF HAMSTERS TO MEF$_1$ STRAIN POLIOMYELITIS VIRUS

Experimental procedure	No animals	Morbidity %	Mortality %
Unilateral adrenalectomy	70	54 3	31 4
Cortisone, 5 mgm × 1	163	84 7	65 6
DOCA implants, NaCl in drinking water	30	80 0	80 0
Castration (recent)	106	67 9	48 1
Testosterone implant	151	49.7	23 2
Controls	126	61.9	32 5

morbidity also rose to 65 1 per cent, again approximating that noted in the earlier portion of the year

Some degree of correspondence could also be observed between mortality from poliomyelitis and testicular activity as expressed by the testicular weight *in mg per 100 gm of body weight*. An inverse relationship between the mortality rate and the degree of testicular maturity appeared to exist. Testicular activity, while determined principally by relative weight of gonads, was periodically checked by sperm counts Thus, animals below 25 gm in body weight [illegible] testes and a mortality rate of 60 34 per cent Animals of an [illegible] r ac- [illegible] oup,

veraging 32.35 per cent. Animals in a higher body weight range (over 55 gm) howed an even greater degree of average testicular activity and, correspondngly, a lower mortality rate (TABLE 7).

Deviation in paralytic morbidity and mortality could also be produced con-

drenalectomy, testosterone overdosage, treatment with chorionic gonadorophin, or pituitary gonadotrophic hormone (TABLE 8)

Effect of Environmental and Hormonal Disturbances upon Brown Fat in the Syrian Hamster

Cortisone treatment resulted in a marked and rapid increase in brown fat olume and weight.[10] The incremented weight was attributable almost exlusively to increase in neutral lipid material, confirmed biochemically and istochemically. Phospholipids were also increased in absolute value, but dereased in relative concentration Water content followed a pattern similar to hospholipids Conversely, bilateral adrenalectomy was followed by brown fat trophy and depletion of sudanophilic material

Discussion and Summary

Studies on the progression of peripherally inoculated poliomyelitis virus in he Syrian hamster, cynomolgus monkey, and mouse indicate that a peculiar orm of connective tissue, descriptively designated as brown fat, serves as the principal extraneural viral proliferation site during the preparalytic and paralytic phases of the disease Virus proliferation in this tissue is reflected microcopically by necrobiotic lesions In the Syrian hamster, another visceral esion, regional myositis, accompanied by significant quantities of intrinsic irus proliferation is also noted Muscle changes appear to be specifically those of the hamster, since it is not demonstrable in other species amenable to poliomyelitis infection Brown fat lesions in the monkey are noted, following inection with numerous strains of type 1 and type 3 poliomyelitis virus

Brown fat is an extremely labile tissue, sensitive to seasonal and temperature changes, stress, and a wide range of experimentally induced endocrine disurbances It reacts, as in the case of cortisone treatment, by expansion of cell diameter, increased lipid content and gross increase in weight The reverse occurs, for example, following adrenalectomy In general terms, brown fat hypertrophy, from whatever cause, coincides with increased susceptibility to poliomyelitis and, conversely, brown fat atrophy with decreased susceptibility It is still uncertain, despite the repeated coincidence of brown fat enlargement and augmented susceptibility, whether both phenomena are causally related or whether they each represent independent end results of still another physiological disturbance as yet undetermined

In an extensive series of experiments, susceptibility also appears to be closely related to alterations, seasonal or induced in adrenal testis equilibrium Heightened susceptibility is correlated with adrenal hypertrophy Increased

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VIREMIA, INVASIVENESS, AND THE INFLUENCE OF INJECTIONS*

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In dealing with a phenomenon that represents a special case of the pathogenesis of poliomyelitis, it is important to emphasize that our knowledge of the fundamental processes is as yet incomplete and that, in preparing to develop the factors that underlie the "provoking" effect of injections, we must dis-

[illegible]

established facts First, in the presymptomatic period of human poliomyelitis infections and of infections in chimpanzees inoculated with invasive strains by virus feeding, virus can be readily isolated from the blood serum [1,2,3] At this time, virus can also be isolated from the feces The distribution of virus in human beings preceding this period of viremia is unknown and probably cannot be directly established However, in chimpanzees sacrificed before any demonstrable virus can be isolated from the blood, virus can be shown to exist in high titer in the feces, in tonsillo-pharyngeal secretions, and in a few lymphatic structures closely associated with the alimentary tract, namely, the tonsils, deep cervical lymph nodes, Peyer's patches, and mesenteric lymph nodes, but in no other viscera that we have examined in three carefully studied animals. Among the tissues that have failed to yield virus are portions of the intestinal walls, the trigeminal ganglia, and samples of practically every internal organ (TABLE 1)

In other words, the distribution of virus in the previremia period suggests primary viral invasion and multiplication in the tonsils and in Peyer's patches of the ileum, with lymphatic spread to local lymph nodes and subsequent spilling over into the blood serum It follows that the tonsils and Peyer's patches may be not only the sources of virus in tonsillo-pharyngeal secretions and feces, but also the sources of at least some of the virus that enters the blood stream Evidence from chimpanzees and from human autopsy cases indicates that, subsequent to viremia, a number of "target" organs may be invaded secondarily by virus from the blood stream These organs include the central nervous system and lymphatic tissues, and, confirming the work of Shwartzman and his colleagues described in this volume the brown fat

Now we know that, in fatal human cases and in chimpanzees experiencing paralytic infection after virus feeding, respectively it is not possible to recover virus consistently from peripheral nervous tissues that might be considered as leading from the alimentary tract to the central nervous system This includes the coeliac ganglia, trigeminal ganglia, and olfactory bulbs In both species,

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc., New York, N. Y.

TABLE 3

EXPERIMENTS ON EFFECT OF PROLONGING INTERVAL BETWEEN INTRAMUSCULAR INJECTION AND INTRAMUSCULAR INOCULATION

Experiments	Interval between injection in right calf with DPT and intracardiac inoculation	Paralytic ratio	Initial right leg paralysis	Initial leg paralysis	Initial arm paralysis	Facial paralysis
1	*1 week*					
	Controls	7/10	0/10	1/10	4/10	3/10
	Injected	8/10	5/10	8/10	0/10	3/10
2	*3 weeks*					
	Controls	8/10	1/10	4/10	4/10	2/10
	Injected	9/10	0/10	3/10	6/10	4/10
3	Controls	4/11	0/11	0/11	4/11	1/11
	Injected					
	1 week	10/11	2/11	4/11	5/11	0/11
	2 weeks	7/11	2/11	3/11	1/11	2/11
	3 weeks	7/11	2/11	3/11	2/11	3/11
Combined experiments	*1 and 2 weeks*					
	Controls	11/21	0/21	1/21	8/21	4/21
	Injected	25/31	9/31	15/31	6/31	5/31
	3 weeks					
	Controls	12/21	1/21	4/21	6/21	3/21
	Injected	16/21	2/21	6/21	8/21	7/21

crease in paralytic rate resulting from intramuscular injections. Animals that did not succumb to poliomyelitic paralysis revealed no visible histological changes in the blood vessels of the segments of the spinal cord corresponding to the injection site, so that our experimental system obviously differs from that described by Trueta and Hodes.[7] TABLE 2 also shows clearly the fact that such intramuscular injections determine the preferential localization of paralysis as well as the production of an increased risk of paralysis. TABLE 3 shows, moreover, that the phenomenon has essentially the same time limitation that has been observed in field studies of the "provoking" effect of intramuscular injections in human beings. In other words, the experimental setup duplicates closely the situation as we can conceive of it in the human population. Thus, the introduction of irritating materials into the muscles at a time when viremia may be presumed to be occurring in large numbers of individuals produces the characteristics of increase of paralytic rate and of selective localization of paralysis during the first two weeks after the intramuscular injection and, to a decreasing degree, subsequently. The increased risk of paralysis has been inferred only from the human field trials but is conclusively shown in the experimental animal.

It becomes of interest, therefore, to analyze this apparently analogous experimental setup to determine, if possible, the mechanism by which the "provoking" effect is accomplished. This, while it may not tell us with certainty the mechanism that obtains in the human species, gives us the only basis for formulating any ideas at all on this subject. I can only summarize briefly several lines of experimentation that we have carried out to determine how it is that the injection of irritating materials intramuscularly both increases the risk of paralysis and determines to a considerable degree the localization of initial paralysis in the experimental animal. First of all, it was observed by us

that two hypotheses could be formulated by which this effect might be obtained, both hypotheses assuming a central role of viremia The hypotheses are as follows: first, the injection of materials intramuscularly, at a time when virus is circulating in the blood stream, could localize such virus into the muscles, whereupon the virus could ascend the nerve fibers, initiate infection in the corresponding part of the spinal cord, and thus produce initial paralysis of the extremity in question. On the other hand, it seemed conceivable that the intramuscular injection merely produced some sort of reflex change in the blood vessels of the corresponding part of the spinal cord, so that circulating virus could more readily penetrate that region and set up an initial infection therein Both hypotheses, it will be observed, fit the fact that initial paralysis and most severe paralysis is localized to the site of the intramuscular injection

My first feeling in this matter was that the more likely hypothesis was that virus was localized by the intramuscular injury and ascended along nerve fibers, as it has been shown to be able to do by earlier experiments. It soon became apparent, however, that the observations were not fitting this hypothesis very well First of all, we observed that the incubation period after intravascular inoculation is almost as short as it is after intracerebral inoculation with the same virus strain. The difference of one to two days theoretically could be explained by the observation that this is the interval required for the development of a viremia of infection and on the assumption that the central nervous system is penetrated by the virus in the blood stream soon after it is produced by the infection Moreover, the incubation period after intravascular inoculation, when a provoking stimulus has been applied in the form of intramuscular injection, is the same as it is when no such stimulus occurs[4] This is most difficult to explain on the basis that the virus in the blood stream is shunted to the injured nerve fibers in the muscle The difficulty arises from the fact that incubation periods, after direct intramuscular inoculation of virus, are much longer than they are after either intracerebral inoculation or intravascular inoculation. Because the mean incubation period after intramuscular inoculation is approximately 12 days with the Mahoney virus, and the incubation period after intracardiac inoculation in animals injected into the calf muscles with nonviral materials is approximately nine and one-half days, we felt that it was not possible to assume that the shunting mechanism could operate.[4]

A further test of this assumption was made by interrupting the sciatic nerve by means of freezing with solid carbon dioxide, a procedure that was shown, some years ago, to prevent effectively the spread to the spinal cord of virus inoculated into the nerve below the frozen segment When such a nerve block is carried out, however, it was found that preferential localization of initial paralysis occurred in the corresponding leg, although no such effect was observed when the sciatic nerve was merely exposed surgically without the interruption by freezing[4] It is difficult to conceive that interruption by freezing would permit circulating virus to localize in the interrupted nerve fibers and, moreover, this procedure produces a localization of poliomyelitic paralysis with approximately the same incubation period as that occurring with intravascular inoculation in uninjured animals We are forced to conclude at the

present time, therefore, that the mechanism of the "provoking" effect of injections or of trauma is to produce a reflex change in vascular penetrability in the vessels of the corresponding part of the spinal cord.

The evidence suggests, therefore, that the variation in "virulence" of virus strains that is readily demonstrated by intracerebral or other intraneural inoculations requires an additional factor for its expression after peripheral forms of inoculation. After virus feeding or vascular inoculation, for example, *the expression of virulence for central nervous tissue is dependent on the variable* "invasiveness" of strains from the blood stream. In nonimmune hosts, the factors of "virulence" and "invasiveness" are ultimately permitted expression by muscular trauma and probably by other predisposing factors that may act by increasing vascular penetrability to virus in local regions of the CNS

[illegible]

result in CNS infection when otherwise appropriate "provoking" stimuli are applied. Such strains have already been described in connection with feeding experiments in human beings and in chimpanzees,[8, 9] and we are now testing our hypothesis of the mechanism of the "provoking" phenomenon, using nonviremic strains in cynomolgus monkeys, rather than the highly invasive strain used in our previous experiments.

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[illegible] glia in

[illegible] infec- [illegible] Am

[illegible] mecha- [illegible]

[illegible] An

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PHYSIOLOGICAL MECHANISMS INVOLVED IN THE LOCALIZATION OF PARALYSIS

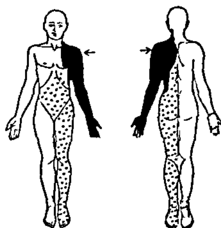
By J. Trueta*
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Information on the characteristics of the viruses responsible for acute anterior poliomyelitis is rapidly accumulating at a time when laboratory studies and epidemiological surveys have provided us with the theoretical information necessary to control the spread of the virus and establish the prophylaxis of the disease, so that it would appear that we are entering the last phase in the struggle against acute poliomyelitis

It may well happen, however, that epidemic poliomyelitis will disappear, leaving the mechanism that determines the localization and severity of the damage to the central nervous system still undetermined The different susceptibility of even siblings exposed equally to apparently the same strain of virus would seem to require some explanation other than simple variation in immunological state of the different members of the family attacked

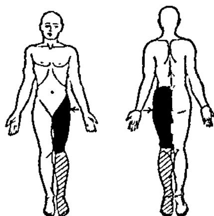
The effect of extraneous factors in determining sites and severity of paralysis was suggested as long ago as 1909 by Hochhaus,[1] who recognized that the disease might follow vaccination, and in 1913, when Wickman[2] called attention to the relationship between muscular exertion and the localization and severity of the paralysis The role of trauma as a localizing factor was recognized by Le Fevre de Arric and Millet[3] in 1929, and that of the immunizing injections, notably those against diphtheria and pertussis, was established by the work of McClosky[4] in Australia, by Geffen[5] in Great Britain, and also by workers in the United States by 1950 Since then, the relationship between injections of many kinds, particularly intramuscular injections, and the onset of paralysis has been confirmed Finally, one must mention the role of tonsillectomy (Francis *et al*, 1942[6]) and pregnancy in favoring the paralytic form of the disease.

We have studied the relationship between these "extraneous" factors and the site and severity of the paralysis in the cases admitted to the hospital of the Nuffield Orthopaedic Centre, Oxford, England, and the injection factor stood out particularly as a real provoking factor in many cases This is particularly frequent in children A few examples will be mentioned to illustrate the point G M, a boy of three years, received a single inoculation of combined antidiphtheria and pertussis vaccine, followed 30 days later by a severe permanent paralysis of the limb that had been injected (FIGURE 1) A B a boy of nine months, had a single inoculation of antidiphtheria pertussis vaccine, followed 15 days later by permanent paralysis of the muscles around the area of injection (FIGURE 2) B N a boy of five and one half years is another similar case followed by permanent paralysis initiated 21 days after the injection (FIGURE 3) Another group of patients had received more than one im



G M, BORN 1950
9 6 53 DIPHTHERIA AND PERTUSSIS INJECTION, LEFT ARM
7 7 53 ONSET OF A P M

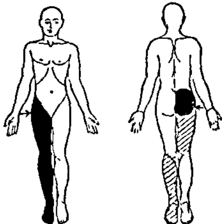
FIGURE 1



A B BORN 26 3 53
18 12 53 DIPHTHERIA AND PERTUSSIS INJECTION, LEFT BUTTOCK
3 1 54 ONSET OF A P M

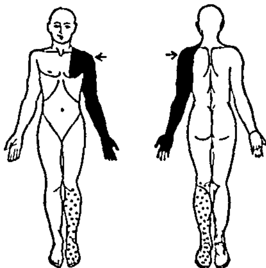
FIGURE 2

munizing injection. These are three examples: G. H, a girl of one year, h an injection against pertussis followed, after little more than two months, by new injection against diphtheria Nine days later, paralysis occurred, a only the shoulder which had been previously injected remained severely a fected (FIGURE 4) P C, a boy of one year, had an inoculation against p



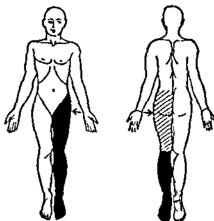
B N, AGED 5½
22 4 53 DIPHTHERIA AND PERTUSSIS INJECTION
13 5 53 ONSET OF A P M

FIGURE 3



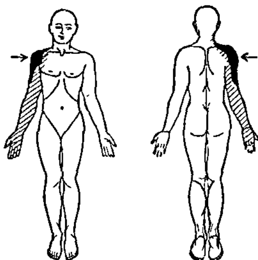
G H, BORN 20 7 52
5 53 INJECTION FOR PERTUSSIS.
16 7 53 INJECTION FOR DIPHTHERIA.
25 7 53 ONSET OF A P M

FIGURE 4



P C, BORN 13 9 51
1 7 52 FIRST INJECTION FOR PERTUSSIS
7 8 52 SECOND INJECTION FOR PERTUSSIS AND DIPHTHERIA.
16 8 52 ONSET OF A P M

FIGURE 5



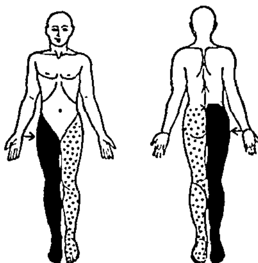
R G, BORN 20 1 53
28 9 53 INJECTION RIGHT ARM FOR PERTUSSIS
12 10 53 INJECTION LEFT ARM FOR DIPHTHERIA.
22 10 53 GENERAL MALAISE AND SORE THROAT.
28 10 53 LOSS OF USE OF RIGHT ARM.
ADMITTED TO HOSPITAL.

FIGURE 6

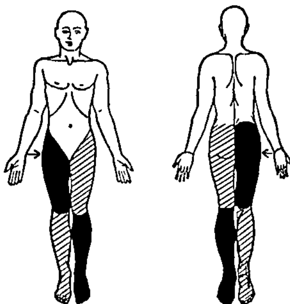
tussis followed just over a month later by a second injection against diphtheria in the same area Nine days later, an attack of poliomyelitis, localized to this area, ensued (FIGURE 5). Of special interest is the case of R. G, a boy of nine months, who had an inoculation in the right arm against pertussis, followed, 14 days later, by an injection against diphtheria in the other shoulder Ten days after this second injection, the child had malaise and sore throat, followed six days later by the loss of use of the right arm, that is, the arm in which the [illegible] se six cases, the injections were all

[illegible] es in which the same relationship [illegible] 'I S, a boy of two years, who had [illegible] nine days later by the onset of permanent flaccid paralysis of the injected leg (FIGURE 7) D S, a girl of four years, had paralytic poliomyelitis 10 days after an injection of T.A B (FIGURE 8)

We had assumed, before the existence of a viremia was as well proved as it is now, that all the different external factors responsible for the site and particular severity of the paralysis could well have as a common cause some



M.S, BORN 27 I 52
22 I·54 I M PENICILLIN COMMENCED
INJECTIONS INTO LEG
31 I 54 ONSET OF A P M



D S, BORN 17 12 49
21 8 53 T A B INJECTION INTO LEG
31 8 53 ONSET OF A P M

FIGURE 8

vascular alteration in the spinal cord, which would increase the permeability of the blood-brain barrier to the virus or, in some way, lower the resistance of the individual motor neurones

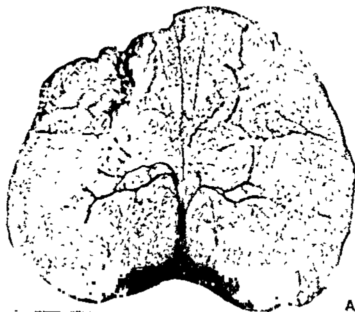
In a paper published in 1954 (Trueta and Hodes[7]) we gave a preliminary report of our work on the vascular patterns of the spinal cord in a variety of circumstances, using Berlin blue alone or associated with barium injected into the arterial circulation as contrast medium.

Briefly we showed in mice:

(1) A marked increase in the number and size of the patent blood vessels of the spinal cord if the animal had been exercised by swimming (FIGURES 9A control and 9B experimental)

(2) Injection of 0 05 ml of 10 per cent formol saline into the right hind limb of a white mouse was followed by paresis of the limb within an hour, and by death after four days Gross inspection of the cord showed very marked vascular engorgement of the vessels of the lumbosacral region on the ipsilateral side Extravasation of blood from the dural vessels of this side was visible on low-power magnification

(3) The same amount of a 1 per cent solution of formol saline in the thigh of a mouse caused death after seven days.


A


B

side of the injection. In FIGURE 10, this is clearly seen. There is a very large engorgement of vessels entering the gray substance on the side of the injected limb, with a large hemorrhage almost within the gray matter on that side, and small hemorrhages on the opposite side In that part of the spinal cord where the hemorrhage begins to subside, the greatest change occurs on the side of the intramuscular injection (FIGURE 11). The vascular engorgement is not only apparent after intramuscular injections of irritants, but may also be elicited by a number of other external agencies We studied the effect of immobilization of a limb in a plaster cast for a fortnight, on the vascular pattern of the cord. Evidence of the type of changes is seen in FIGURES 12A and 12B Larger vascular caliber appeared on the side of the cord corresponding to the immobilized limb

In an attempt to determine whether the same susceptibility to change was shown by the vessels of the spinal cord after subcutaneous injections of irritants, we injected two groups of mice with 0 05 ml croton oil 1 in 10, one group intramuscularly and the other group subcutaneously. FIGURE 13 shows sections of the white columns of 10 animals injected intramuscularly, and it shows that, from the first, the mortality was greater than in the series injected subcutaneously. By the seventh day, all the animals injected intramuscularly had died, while only 1 out of 10 of those injected subcutaneously had died By the 13th day, the mortality of the subcutaneously injected animals had risen to its maximum of 6 in every 10 Considering the great difficulty of

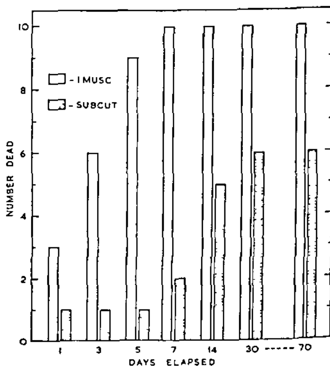


FIGURE 13

				INTRAMUSC.		SUBCUTAN	
EXP'T NO.	SITE OF INJ.	DIL.	DAYS OF EXP'T	AV. DAY OF DEATH	NO. DEAD	AV DAY OF DEATH	NO DEAD
1	THIGH	1 10	77	3 2	10	10 3	6
2	"	1 20	59	14 6	8	25 1	7
"	GAST	"	"	22 0	4	25 0	1
"	ARM	"	"	14 5	2	27 7	3
4	"	1 10	42	15 0	4	34 0	2
5	THIGH	"	35	12 7	6	7 0	1
TOTAL DEAD				34		20	
PER CENT DEAD				56 6		33 3	
AV DAY OF DEATH				11 8		21 0	

Figure 14

keeping the injected mass subcutaneously in such a small animal as the mouse, the difference in results seems to be of some significance It was thought interesting to study the different rates of survival among mice injected either intramuscularly or subcutaneously, in several regions of the body In the thigh the intram- [illegible]

jected in the calf, the total number of deaths was 4 in every 10 following the intramuscular injection of the 1-in-10 solution and only 1 among 10 after the subcutaneous injection In the arm 0.05 m' of 1 in 20 solution of croton oil caused death after an average survival of 14 5 days, intramuscularly, while a 27 7 day survival was obtained following the subcutaneous injection of the same amount of solution A new series of thigh injections caused the death of 6 out of 10 mice after intramuscular injection and of only 1 in 10 following subcutaneous injection It may thus seem that injections in the thigh affect the animals more severely than those either in the calf or in the arm, the least

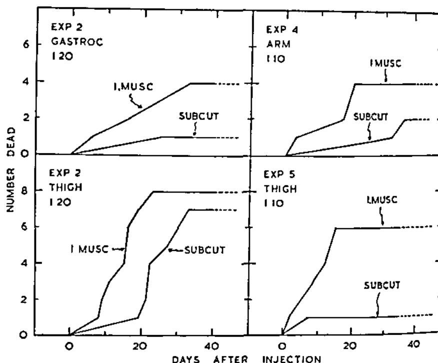


FIGURE 15

dangerous of all apparently being the subcutaneous injection in the arm FIGURE 15 shows again the differing susceptibility following intramuscular or subcutaneous injection and is self-explanatory. In these experiments, no positive data as to the cause of death were obtained, but the postmortem examination of several mice made us suspect that the vascular changes of the spine had some part, at least, in the death of the animal.

The possible clinical implications of these findings may be of sufficient interest to encourage their early publication Alternative methods for introducing therapeutic and prophylactic substances into the body other than intramuscular injections, particularly in the thigh, are to be recommended

Acknowledgments

I should like to thank Doctor M Agerholm and R. Edwards, A. Mann, and W Charles for their help in the prosecution of this work. I am grateful to *The Lancet* for allowing me to publish FIGURES 9 to 12.

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Part III. Poliomyelitis Virus Variation

POLIOMYELITIS VIRUS VARIATION*

By Jerome T. Syverton
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It is fitting that the Conference on the Biology of Poliomyelitis upon which this monograph is based should have focused attention on variation of the causative virus. Let us first consider variation in its broad sense. As Findlay (1936) observed, a change in character of a virus is variation: for example, a change in virulence, in host range, or in route of infectivity. Variants of poliomyelitis virus have been recognized recurrently, under natural and experimental conditions, since the discovery of the virus by Landsteiner and Popper in 1909. Flexner and Clark, in 1911, observed variation in the severity of disease induced in monkeys by primary transfer of 10 human cords. They applied the apt description "races of the virus." Flexner, Clark, and Amoss (1914) found similar variants on serial passage of single strains of virus. Later these differences were so emphasized by selection (Flexner and Amoss, 1924) that strains could be characterized as "immunizing" or "killing." The observations of strain variation in pathogenicity were difficult to interpret, particularly since the protection conferred by infection with a mild immunizing strain of virus might be obscured when the host was challenged with an immunologically distinct strain.

The problem was resolved in part by the recognition of immunological differences among strains. The names of Burnet and Macnamara (1931), Weyer (1931), Flexner (1932), Paul and Trask (1933, 1935) and others are associated [illegible]

[illegible] sis, New York, N. Y.

The paths of the geneticist and the microbiologist have so converged in recent years that, for each, the fundamental unit of study is a self replicated macromolecule, the inheritance of characters, whether of microbes or men, now must be understood in terms of this unit. We shall not hesitate, then, to apply the terms of classical genetics to the biology of variation in an animal virus.

Poliomyelitis virus exists as a collection of particles. It is inconceivable that this population of replicated units should represent a suspension of identical particles. The minor differences from particle to particle, that on long continued propagation and selection can give rise to strain differences, may be called variations. These are reversible. Major differences that are sudden in appearance, infrequent in occurrence, and stable in transmission, are analogous to mutations in higher forms and may be so named. These changes are rarely reversed. Poliomyelitis virus is propagated experimentally in susceptible cells

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc., New York, N. Y.

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[illegible]
LAU [illegible]
PAU [illegible]
WEY [illegible]

EVOLUTION OF THE CELLULAR LESION INDUCED BY POLIOMYELITIS VIRUS *IN VITRO* AS STUDIED WITH PHASE CONTRAST MICROCINEMATOGRAPHY

By G. Barski, R. Robineaux, and M. Endo*
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In a preliminary note,[1] we reported some observations made on poliomyelitis virus-infected cells *in vitro*, fixed and colored by different methods, namely Giemsa stain, hematoxylin-eosin, pyronine-methyl green after Unna Pappenheim, Feulgen, and alkaline phosphatase cytochemical reaction after Gomori. These methods, in our hands, enabled us to describe the specific lesion as follows.

An eosinophilic area, at first rather undetermined but soon more precisely outlined, appears in the cytoplasm in the vicinity of the nucleus. This central mass pushes the cytoplasm and nucleus aside. At this stage, the general basophilia [illegible] ys connected with a [illegible] se reaction. Meanwhile [illegible] *the nucleoli become* distorted but remain highly pyroninophilic. In the marginal cytoplasmic area, deeply stained basophilic and Feulgen positive granules often appear. During the following phase, the colorability of the central mass decreases and so does the general basophilia of the cytoplasm. The cell progressively clears up and seems to lose its cytoplasmic content.

We observed the same pattern of lesions in various types of cells cultivated *in vitro*: human adult and embryonic fibroblasts, human and monkey kidney cells.

The purpose of the present study was a continuous observation of living infected cells by means of a moving camera connected with a phase-contrast microscope.

Material and Methods

The fibroblasts used for this study originated from human tonsils and, before their use for experimentation, were maintained by serial passages in roller tubes for two or three months. These cells are much larger than average skin muscle fibroblasts. When cultivated, they appear well spread, regular in shape, and remarkably resistant to different factors of nonspecific degeneration. Their sensitivity to poliomyelitis virus *in vitro* has previously been established.[2]

For direct examination and recording, the cellular material was explanted by the hanging-drop technique in a complete medium, providing the cells, as seen in noninfected controls, with optimal conditions for development.

Before transplantation, the cells were incubated for 30 minutes at room temperature with a high titer (10^{-6}) virus suspension. MEF1 type II and Mahoney type I strains were used, and no difference in the appearance of cellular lesions was noted for either type of the virus.

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Observations were carried at 36 to 37° C. and 30 to 31° C. respectively. The evolution of the cellular lesion was the same at both temperatures, but slower at 30 to 31° C As the general condition of the control cells was better at 30 to 31° C., we preferred using the lower temperature

Observations

The observation* of living polio-infected culture cells with the phase contrast microscope generally corroborates observations previously made on fixed and stained material

In the early stage of infection, around the 20th hour at 30 to 31° C, the most conspicuous changes take place in the cytoplasm in its perinuclear zone more or less homogeneous mass gradually increases in the center of the cell Its texture is different, as were its staining properties, from that of the surrounding normal cytoplasm Its content remains almost motionless while, in the cytoplasm driven aside toward the cell periphery, signs of activity are still obvious, and, most strikingly, mobile, filamentous mitochondria persist in it for a long time. Later on, the diseased and already damaged cell displays a conspicuous dynamic activity showing a bubbling phenomenon quite similar to that observed in dividing cells in metaphase As we have seen previously, this stage is characterized by a general basophilia which becomes as intense as it is in cells during mitosis in control cultures.

After the "bubbling" stage, the cell comes to a standstill, the outer layer of the cytoplasm showing intense vacuolization resulting finally in the gradual disintegration of the outline of the cell

This pattern of lesions is never seen in the control noninfected cells, even when they display nonspecific degeneration and death.

Discussion

The question about the nature of the dense and motionless mass appearing and developing in the center of poliomyelitis-infected cells in tissue culture cannot as yet be answered There is no evidence that it represents the site of the virus synthesis or of its maturation, though certain data show that, for polio, this synthesis is accomplished primarily, if not exclusively, in the cytoplasm [4, 6]

As far as the staining properties of this area are concerned, it may be recalled that segregation of the cytoplasmic and/or nuclear content into acidophilic and basophilic elements may be looked upon as a more general phenomenon in virus-infected cells (*e g*, vaccinia-variola, herpes, rabies) Obviously, the appearance of these "segregated" elements can be due to the accumulation not only of the virus protein itself, but of other protein material also, resulting from the abnormal metabolism in the infected cell

It is generally admitted that, in *in vitro* cultures, the elimination of the virus by poliomyelitis-infected cells occurs before the final destruction of the cell at a time when the cells still display an active metabolism [3] The mechanism of this release has remained unknown According to the quantitative data given

* Frames from this cinematographic study are given in another publication [2]

by Dulbecco and Vogt,[6] by Ackermann, Rabson, and Kurtz[3] and, more recently on single isolated cells, by Lwoff, Dulbecco, Vogt, and Lwoff,[8] this release is gradual and different from the bursting of bacteriophage-infected bacteria. As reported above, during the period corresponding to the production and the release of the virus, the cell shows, first, active movements, then fast progressing vacuolization and dissolution of the outer cytoplasm. On the whole, the moving pictures of this activity have allowed us to discover a strong dynamic activity associated with a high morphological instability at the outer limits of the infected cell.

These facts suggest certain mechanisms of virus transfer from a diseased but still living cell to the surrounding medium, in which the proper cell activity may play a part.

Summary

Cultures of adult, human fibroblasts of relatively large size and high resistance to nonspecific degeneration were infected with types I or II poliomyelitis virus and submitted to continuous observation by means of phase-contrast microcinematography. A central dense and motionless mass regularly appeared in the cytoplasm. As this mass increased in size at the expense of the cytoplasm, the nucleus was pushed to the periphery of the cell, but the remaining cytoplasm continued to exhibit signs of vital activity. The "bubbling" and vacuolization of the marginal cytoplasm observed in the infected cells are considered as representing a possible mechanism for the release of virus before the ultimate destruction and death of the cells.

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EXPERIMENTALLY PRODUCED VARIANTS OF POLIOMYELITIS VIRUS COMBINING *IN VIVO* AND *IN VITRO* TECHNIQUES

By C. P. Li, M. Schaeffer, and D. B. Nelson

Communicable Disease Center, Public Health Service, United States Department of Health, Education, and Welfare, Montgomery, Ala

Recent technologic advances have permitted a variety of laboratory manipulations that have made possible the selection and study of several variants of each of the three types of poliomyelitis virus. Such studies are not only revealing new and important information on the biologic and genetic constitution of the viruses, but are also responsible for the development of methods by which variants with specifically selected characteristics may be more readily isolated and "fixed."

Among the many possible applications there is, of course, the desire to isolate highly antigenic but at the same time avirulent or nonneurotropic variants of poliomyelitis virus for use in the development of live virus vaccines, following a pattern similar to that so successfully demonstrated in yellow fever.

Much of the effort that has been cast in this direction by us and others has resulted in the derivation of highly promising data.[1-6] The information presented in this report includes portions of a continuing study concerned with the experimental production of variants of poliomyelitis virus, using *in vivo* techniques in rodents, monkeys, and chick embryos. The spontaneous appearance of variants following the *in vitro* cultivation of virus will also be described.

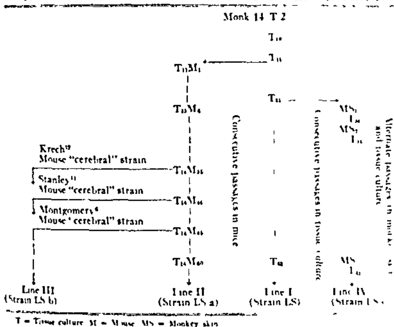
That the passage of poliomyelitis virus through rodents may result in a reduction of its virulence for monkeys has previously been observed.[2,7,8] Recently, Li and Schaeffer[9] have shown that strains of virus, types 1 and 3, may lose their virulence for monkeys following passage in monkey testicular tissue culture. Simultaneously, these variants may acquire other biologic characteristics. A further variation in type 1 virus was also noted after several alternate monkey skin passages *in vivo* and monkey kidney tissue cultures.[6] Addit [illegible] different tissues exist in th [illegible]

[illegible] ts were begun with a number of strains of type 1, including the Mahoney strain. The latter after a few passages in monkey testicular tissue culture, exhibited newly acquired characteristics. Two characteristics that became apparent somewhere between the 6th and 11th passages were the ability of the variant to infect mice via the intraspinal route and its loss of infectivity for monkeys via the intracerebral route. Further passage lines were carried out with this variant. These lines are shown in TABLE 1.

Line I, therefore, is the direct derivative of the highly virulent Mahoney strain that no longer infects monkeys following inoculation by any route except in an occasional monkey when groups of 6 or 12 are inoculated directly

TABLE 1

LINES OF PASSAGE OF POLIOMYELITIS TYPE 1 VIRUS RESULTING IN VARIANTS



T = Tissue culture M = Mouse MS = Monkey skin

into the lumbar portion of the spinal cord This spinal variant has been designated as substrain LS

Line II is a variation of Line I, maintained by consecutive intraspinal passages in mice with slow but definite increase in virulence for mice This line is designated as substrain LS-a and, in most respects, is similar in behavior to LS

Line III (LS-b) is derived from Line II and has been "adapted" to infect mice via the cerebral route by Stanley,[11] Krech,[12] and in our laboratory [4]

Line IV, substrain LS-c, which is of primary interest at present, was derived from Line I by alternate passages in monkey skin and monkey kidney tissue culture This was accomplished by intracutaneous injection of the tissue cultured virus, the skin biopsied after three to six days, and reinoculated into tissue cultures Between the second and seventh of such alternate passages the virus was found to be no longer paralytogenic for mice or monkeys by any route, including the intraspinal

A total of 250 mice and 75 monkeys (17 cynomolgus and 58 rhesus) have been inoculated intraspinally to date with LS-c virus, most of them with undiluted tissue culture fluid containing 10^{-7} tissue culture doses of virus, but some received the culture diluted 1:10, and diluted 1:100. None of these animals has shown clinical signs of infection, nor have histopathologic changes been ob-

TABLE 2

REACTIONS OF VARIANTS OF POLIOMYELITIS VIRUS, TYPE 1, DERIVED FROM MAHONEY STRAIN

Variant	Host range						
	Tissue culture (monkey kidney)	Monkey			Mouse		
		Intra-cerebral	Intra-spinal	Parenteral	Intra-cerebral	Intra-spinal	Intra-venous
Mahoney	+	+	+	+	−	−	−
LS	+	−	+	−	−	+	ND
LS-a	+	−	+	−	−	+	ND
-b	+	−	+	−	+	+	+
-c	+	−	−	−	−	−	−

ND = not done

served in the central nervous system (CNS) of these animals, sacrificed 14 or more days following injection

The basis for differentiation of the variants which are all neutralized by antiserum prepared against the parent Mahoney strain is presented in TABLE 2, which illustrates their distinct reactions in different hosts

A passage line of strain LS-c that has exhibited no affinity for cells of the CNS by the tests conducted thus far was used to immunize a group of 12 monkeys that received, intradermally, 0 5 ml of undiluted tissue culture fluid containing 10^{-7} tissue culture doses per ml Only one of these monkeys exhibited evidence of mild poliomyelitic infection when challenged intramuscularly with the virulent Mahoney strain, while 6 of 11 controls succumbed with prostrating paralysis.

Two chimpanzees given 0 25 ml of this virus intracutaneously developed no signs of infection, had no viremia, nor did they shed virus in their stools. They responded, however, with antibody titers of 1:32 and 1:64 in neutralization tests against 100 tissue culture doses The duration and protective effect of this antibody response remains to be determined

Experiments w ... adapted to mice by intra ... substrain LH ... intraspinal passages, has increased in virulence for mice and retained, to some extent, its pathogenicity for monkeys After 72 mouse passages, the LH strain was passed into monkey testis tissue culture Following one such culture passage, only two of eight mice inoculated were paralyzed although the tissue culture titer was 10^{-4} Repeating this, with material from the 72nd mouse passage, the virus propagated again through only a single monkey testis tissue culture failed to infect any of eight mice inoculated intraspinally. Subsequently, none of 106 mice has developed symptoms of poliomyelitis when inoculated with further subcultures of this virus in monkey testis tissue cultures Included in this group are mice inoculated with material receiving from two to six monkey kidney tissue culture passages after the 15th testis tissue culture

TABLE 3
PASSAGES AND TITERS OF TYPE 3 (LEON) POLIOMYELITIS VIRUS

Passage*	Titer in T. C.	Material inoculated I.C. into mice			
		Undil.	1:10	1:100	1:1000
M_{14}			8/11		
M_{15}–M_{75}			232/305		
M_{80}	$>10^{-4.4}$		6/8	2/8	1/8
M_{80}–M_{99}			127/156		
M_{101}	$>10^{-4.6}$		8/9		
M_{102}–M_{111}	$>10^{-4.6}$		90/99		
$M_{21}T_1$		2/8			
$M_{21}T_1$ (repeat)		0/8			
$M_{21}T_4$	$10^{-4.4}$	0/10			
$M_{21}T_{16}$	$10^{-4.4}$	0/40	0/12	0/12	
$M_{21}T_{16}K_3$		0/12	0/12		
$M_{21}T_{16}K_4$		0/20	0/20		
$M_{21}T_{16}K_6$	$10^{-4.4}$	0/12	0/12		
$M_{101}K_1$		19/20	2/10	1/10	
$M_{101}K_2$	$10^{-4.4}$	19/20	16/20	6/20	
$M_{101}K_3$		6/9			

* Abbreviations:
M = mouse intraspinal passage
T = monkey testis tissue culture passage
K = monkey kidney tissue culture passage
Number indicates number of successive passages made

passage. This variant, therefore, is regarded as differing from the LH spinal variant and is designated as substrain LH-a.

Two attempts were made to duplicate these results using monkey kidney tissue culture in place of testis tissue culture. As illustrated in TABLE 3, when strain LH in mouse passage 101 was passed in monkey kidney tissue cultures there was no evidence of alteration of mouse pathogenicity, even after five such passages.

In addition to loss of mouse virulence, substrain LH-a appears also to have lost monkey pathogenicity by the intraspinal route. Of six monkeys thus inoculated, only one showed mild symptoms and minor histopathologic changes confined to the site of inoculation. Of 12 monkeys inoculated intraspinally with this strain of virus after 17 testis tissue culture passages followed by 3 alternate monkey skin and tissue culture passages, none had clinical or histopathic signs of infection.

Another interesting difference between the mouse-maintained substrain LH and the testis tissue culture altered variant LH-a is their different cytopathic reactions observed when they are cultured in monkey kidney cell culture preparations. Strain LH-a displays a reaction that is more or less typical of cytopathogenic changes observed with poliomyelitis viruses. The first signs of degeneration nearly always appear 24 hours after inoculation. The sheets of cells are quite uniformly attacked and, after degeneration is well under way, the surrounding clumps of tissue then begin to show degenerative changes. Destruction of the cells in a tube is usually complete after three to five days (FIGURE 1).

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LS-a	+	−	+	−	−	+	ND
-b	+	−	+	−	+	+	+
-c	+	−	−	−	−	−	−

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Two chimpanzees given 0 25 ml of this virus intracutaneously developed no signs of infection, had no viremia, nor did they shed virus in their stools They responded, however, with antibody titers of 1:32 and 1:64 in neutralization tests against 100 tissue culture doses The duration and protective effect of this antibody response remains to be determined.

Experiments with type 3 virus Type 3, Leon poliomyelitis virus was adapted to mice by intraspinal passage by Li and Habel,[13] and has been designated as substrain *LH*. This virus, maintained in this laboratory by consecutive mouse intraspinal passages, has increased in virulence for mice and retained, to some extent, its pathogenicity for monkeys After 72 mouse passages, the LH [illegible] culture

passage, the virus propagated again through only a single monkey testis tissue culture failed to infect any of eight mice inoculated intraspinally Subsequently, none of 106 mice has developed symptoms of poliomyelitis when inoculated with further subcultures of this virus in monkey testis tissue cultures Included in this group are mice inoculated with material receiving from two to six monkey kidney tissue culture passages after the 15th testis tissue culture

TABLE 3
PASSAGES AND TITERS OF TYPE 3 (LEON) POLIOMYELITIS VIRUS

Passage*	Titer in T.C.	Material inoculated I.S. into mice			
		Undil	1/10	1/100	1/1000
M_{34}			8/11		
M_{34}–M_{72}			232/305		
M_{69}	$>10^{-3.4}$		6/8	2/8	1/8
M_{69}–M_{74}			127/156		
M_{101}	$>10^{-3.0}$		8/9		
M_{101}–M_{111}	$>10^{-3.5}$		90/99		
$M_{72}T_1$		2/8			
$M_{72}T_1$ (repeat)		0/8			
$M_{72}T_4$	$10^{-5.0}$	0/10			
$M_{72}T_{16}$	$10^{-6.0}$	0/40	0/12	0/12	
$M_{72}T_{15}K_2$		0/12	0/12		
$M_{72}T_{15}K_3$		0/20	0/20		
$M_{72}T_{16}K_4$	$10^{-4.0}$	0/12	0/12		
$M_{101}K_1$		19/20	2/10	1/10	
$M_{101}K_3$	$10^{-4.5}$	19/20	16/20	6/20	
$M_{101}K_5$		6/9			

* Abbreviations
M = mouse intraspinal passage
T = monkey testis tissue culture passage
K = monkey kidney tissue culture passage
Number indicates number of successive passages made

passage. This variant, therefore, is regarded as differing from the LH spinal variant and is designated as substrain LH-a.

Two attempts were made to duplicate these results, using monkey kidney tissue culture in place of testis tissue culture. As illustrated in TABLE 3, when strain LH in mouse passage 101 was passed in monkey kidney tissue cultures there was no evidence of alteration of mouse pathogenicity, even after five such passages.

In addition to loss of mouse virulence, substrain LH-a appears also to have lost monkey pathogenicity by the intraspinal route. Of six monkeys thus inoculated, only one showed mild symptoms and minor histopathologic changes confined to the site of inoculation. Of 12 monkeys inoculated intraspinally with this strain of virus after 17 testis tissue culture passages followed by 3 alternate monkey skin and tissue culture passages, none had clinical or histopathic signs of infection.

Another interesting difference between the mouse-maintained substrain LH and the testis tissue culture altered variant LH-a is their different cytopathic reactions observed when they are cultured in monkey kidney cell culture preparations. Strain LH-a displays a reaction that is more or less typical of cytopathogenic changes observed with poliomyelitis viruses. The first signs of degeneration nearly always appear 24 hours after inoculation. The sheets of cells are quite uniformly attacked and, after degeneration is well under way, the surrounding clumps of tissue then begin to show degenerative changes. Destruction of the cells in a tube is usually complete after three to five days (FIGURE 1).

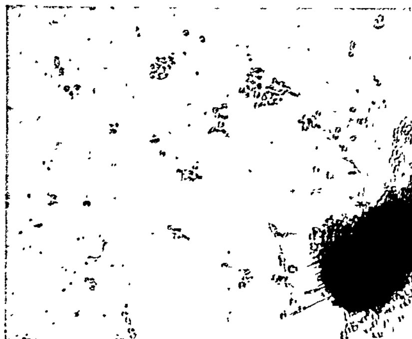


FIGURE 1 Typical cytopathogenic effect in monkey kidney tissue culture three days after inoculation with type III (Leon) substrain LH a poliomyelitis virus ×380

With strain LH, however, the first stages of degeneration begin, or at least ... progresses, the ... of apparently ... visible before 120 hours after virus is added and the total reaction is seldom complete before 10 days By this time, however, a certain amount of nonspecific degeneration begins to set in, rendering end-point determinations of virus titrations difficult

Preliminary experiments with the cultivation of virus in skin grafts on the chorioallantoic membrane of chick embryos. The successful cultivation of poliomyelitis virus in monkey kidney and skin tissue fragments grafted onto the chorioallantoic membrane (CAM) of chick embryos has been reported previously[10] Among the many possibilities afforded by such a system is its use as an intermediate step in the adaptation of the virus types to the chick tissues In addition, it might serve as a means of adapting suitable virus strains to the extraneural tissues of primates and humans A nonneurotropic poliomyelitis virus capable of multiplying in monkey or human skin would have particularly ... een ... AM ... rom the abdomen of normal cynomolgus monkeys These were trimmed of all visible excess fat and subcutaneous tissue and cut into two mm. cubes. Five

FIGURE 2 Atypical spotty degeneration of cells in monkey kidney tissue culture seven days after inoculation with type III (Leon) substrain LH poliomyelitis virus ×380

FIGURE 3 Monkey kidney fragment engulfed by the CAM seven days after grafting ×133

or six such fragments suspended in tissue culture growth medium were then deposited with a pipette onto the dropped CAM of the seven-day-old windowed chick embryo. The window was covered, the egg incubated for 24 hours, after which about 0.5 ml. of virus suspension was deposited on the surface of the grafted tissue. After further incubation for about seven days, the grafted area and the allantoic fluid were harvested and titrated in monkey kidney tissue [illegible]

through 19 consecutive skin graft or occasional allantoic fluid to skin-egg passages. Skin-graft titers have remained relatively high throughout the series of passages, while allantoic fluid titers originally less than $10^{-0.5}$, though fluctuating with passage, have tended to increase about two to three logs in virus content.

That multiplication of virus occurs in this system is evidenced by the high

TABLE 4

PASSAGE OF TYPE 1 POLIOMYELITIS VIRUS IN MONKEY SKIN GRAFTED ON CAM OF EGGS

Passage strain LD-c $MS_{10}T_{10}$		Tissue culture titer: Skin graft	Tissue culture titer: Allantoic fluid
E_{1a}*		$>10^{-6.5}$	ND
E_{2a}		$10^{-6.0}$	ND
E_{3a}		$10^{-6.0}$	$<10^{-0.5}$
E_{4a}		$>10^{-4.0}$	ND
E_{5a}		$10^{-3.5}$	$<10^{-0.5}$
E_{6a}		$10^{-3.5}$	$<10^{-0.5}$
E_{7a}		$10^{-4.0}$	$10^{-0.5}$
E_{8a}		$10^{-4.0}$	$<10^{-0.5}$
E_{9a}	Allan fl → E_{10b}	$10^{-5.5}$	$>10^{-2.5}$
E_{10a}	Allan fl → E_{11b}	$10^{-7.0}$	$10^{-4.0}$
E_{10b}†		$10^{-7.0}$	$10^{-1.5}$
E_{11a}		$10^{-5.5}$	ND
E_{11b}		$10^{-6.0}$	$10^{-1.0}$
E_{12a}		$10^{-6.0}$	$10^{-1.5}$
E_{13a}	Allan fl → E_{14b}	$10^{-4.0}$	$10^{-1.0}$
E_{14a}		$10^{-6.0}$	$10^{-4.0}$
E_{14b}	↓ E_{15b}	$10^{-5.5}$	$10^{-2.5}$
E_{15b}	Skin graft → E_{15a}	$10^{-5.0}$	$10^{-3.0}$
E_{15a}	Allan fl → E_{16b}	$10^{-6.0}$	$10^{-2.0}$
E_{16b}		$10^{-6.0}$	$10^{-3.0}$
E_{17a}		$10^{-5.0}$	$10^{-3.0}$
E_{18a}		$10^{-6.0}$	$10^{-3.0}$
E_{19a}		$10^{-5.0}$	$10^{-3.0}$

* a = skin graft to skin graft passage
† b = allantoic fluid to skin graft passage
ND = not done



FIGURE 4 Section of monkey skin graft showing the CAM surrounding the epidermis ... epithelial layer is partly split away from the membrane due to tissue shrinkage in the ...

titers maintained in the skin graft after 19 consecutive passages and the increase of virus by two to five logs in the graft seeded with allantoic fluid as passage material At which site, or in which cells, the virus multiplies has as yet not been determined When the skin graft "takes," the implant becomes rapidly engulfed by the CAM, and there is little necrosis or degeneration Proliferation of the connective tissue and invading chick capillaries, as evidenced by nucleated erythrocytes, are seen penetrating into the grafts and establishing circulation (see FIGURES 3 and 4) When virus is added to such grafts, no cytopathogenic reactions are recognized in either the donor or recipient cells, although virus proliferation apparently occurs either in or at the site of the graft Virus present in other areas of the embryo is probably disseminated there by diffusion and spillage

Discussion

The accomplishments thus far are indeed short of the desired goal, since there are not as yet satisfactory criteria for prescribing the safe and effective utilization of avirulent virus variants for the immunization of humans against poliomyelitis There are, however, some aspects of this study worthy of emphasis ... method of elicit- ...

tive selector of variants that otherwise might have been missed Observations

concerned with this characteristic, noting first its apperance, then its disapperance, have been responsible for the isolation of strains of types 1 and 3 virus, which are also avirulent for monkeys. It is interesting, however, that the changes occurred during growth in tissue culture and not in the mice

Noteworthy, also, is the fact that monkey testis tissue cultures, which are generally less favorable than kidney cell cultures for poliomyelitis virus proliferation seem to favor the emergence of avirulent mutants. A single passage [illegible]

gation of the role of various tissue culture media in this respect

The application of additional techniques such as the Dulbecco plaque method[14] and others will exhibit more of the inherent characteristics of the known virus strains. Moreover, the potentials of current techniques and those of newly devised ones should serve to uncover the existence of hitherto unrecognized strains possessing important and desirable properties Some of these will undoubtedly be useful in the production of efficient live virus vaccines

Summary

Methods combining *in vitro* and *in vivo* techniques are utilized for the isolation and characterization of avirulent variants of types 1 and 3 poliomyelitis virus The pathogenicity for mice via the intraspinal route was used as an important criterion for detecting their apperance, although the variants occurred spontaneously in monkey testis tissue cultures.

A series of preliminary experiments is described, utilizing monkey skin tissues grafted onto the chorioallantoic membrane of chick embryos for the successful proliferation of type 1 virus This investigation is being pursued with a view toward adaptation of the virus either to the chick tissues or monkey skin

The significance of these studies and their application toward the development of a live virus vaccine are discussed.

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EXPERIMENTALLY PRODUCED POLIOMYELITIS VARIANT IN CHICK EMBRYO

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The cultivation of the MEF_1 strain of type 2 poliomyelitis virus in the developing chick embryo has been reported in previous papers.[1, 2] This communication presents an amplified study of series R-382 of the chick-embryo adapted strain, and provides evidence of the modification of the pathogenicity of the virus for primates and maintenance of its antigenic stability after chick embryo adaptation.

Materials and Methods

Virus. The origin of series R-382 has already been described.[1] Figure 1 illustrates the history of the passages, starting with virus from hamster brain and cord material that was passaged in mice and then adapted to the chick embryo. The method of passage was similar to that followed previously,[1] except that the dose of virus for egg inoculation after the 40th generation was 0.2 ml. instead of 0.6 ml., and the eggs were incubated for five days rather than seven. For the exposure of monkeys and chimpanzees, suspensions of chick embryo material were used representing egg passages between the 22nd and 104th transfers, as specified in the descriptions of the several experiments.

Chimpanzees and monkeys. Chimpanzees six to nine months old and rhesus and cynomolgus monkeys weighing six to nine pounds were used.

Neutralization tests. For antibody determination by mouse neutralization tests, MEF_1 virus from the 64th hamster passage was used.[3] A standard Lansing type hyperimmune serum routinely employed in this laboratory[4] served as control.

Methods of infection. The primates were fed the virus mixed with cream through a rubb

made in the tha

suspension M

chick-embryo suspension in the space between the first and second lumbar vertebrae. Intramuscular inoculation of chimpanzees and monkeys was made into the muscles of the hind legs and, for the intravenous inoculation of monkeys, 2.5 ml. of 20 per cent chick-embryo suspension mixed with an equal amount of heparin solution containing 20 units per ml. was injected in the femoral vessels.

Clinical observations and pathological

least one month following exposure to

Body temperatures were taken daily.

If an animal died, either with or without definite signs of paralysis, the central nervous system was examined histopathologically. Extensive histological exam-

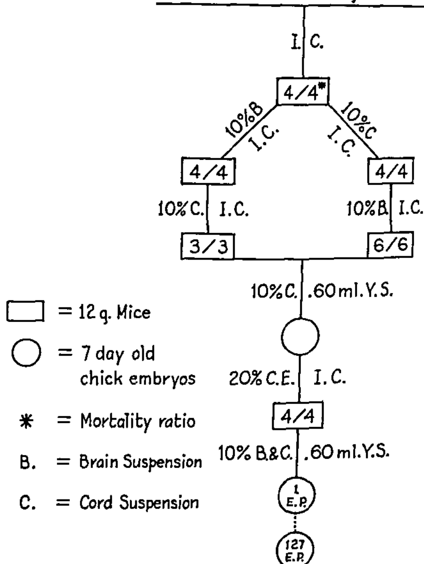


FIGURE 1 Origin of the MEF₁ chick embryo adapted strain (series R-392)

ination was also done on the spinal cords of all symptomless monkeys injected intracerebrally, intraspinally, and intravenously, at the end of the one-month observation period All animals were bled for antibody determinations before the experiments began, and at intervals thereafter

Histopathological examinations of the central nervous systems of monkeys were performed on celloidin sections stained by the Nissl method Cellular losses in the anterior horn of the spinal cord were estimated by Bodian s procedure[5]

Tissue cultures Stationary test tubes were used, each containing 1 0 ml of trypsinized renal epithelial cells of monkeys[6] in medium 199, with penicillin and streptomycin added to a final concentration of 100 units and 0 1 mg respectively per ml. Each tube received an inoculum of 0 1 ml of the material to be tested Three or four tubes were inoculated per sample After incubation at 37° C for three or four days, the cultures were examined for cytopathogenic effect and graded according to the cellular destruction The supernatant fluids were then harvested, pooled by samples and transferred to new tissue culture tubes. Material from each pool was also titrated by tenfold dilutions intracerebrally in mice.

Experimental

Chick embryo Series R-382 virus has so far been passed through 133 chick embryo generations without any difficulty, demonstrating the ready adaptation of the MEF_1 strain to this host FIGURE 2, which gives LD_{50} mouse titers for each 8th passage between the 8th and the 126th transfers, shows that the virus reached and maintained a titer of log 4 5 to 6 0 after the 50th serial passage.

Pathogenicity for the chick embryo Two experiments were performed in order to study the course of infection and to investigate whether any pathological changes occurred in the infected developing chick embryo In the first experiment [illegible]

[illegible]

same conditions Starting the first day after inoculation, blood, yolk sac, [illegible]

[illegible]

viously had been found to contain traces of virus only Pathological studies were made of live embryos from both infected and control groups The results of this experiment, with special reference to the pathological findings, have already been described,[7] and are only briefly summarized here the virus infection interfered with the growth of the embryo, occasionally causing death, specific lesions were observed in 96 per cent of the infected embryos upon histological examination of the nervous system between the 5th and 17th days after inoculation, and myocarditis, probably caused by the poliomyelitis infection, was present in 6 out of 26 embryos showing specific lesions

In the second experiment, 105 seven-day-old embryos were infected with high (113th) egg passage material, each embryo receiving 173,320 mouse LD_{50} doses of virus Incubation was carried out as before This time no pathological investigations were made The course of the embryonal infection has

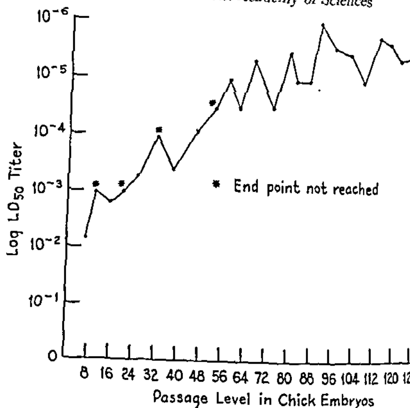


FIGURE 2 LD_{50} mouse titers of MEF_1 strain (series R-382) at various passage levels in chick embry

been visualized in FIGURE 3 by plotting the mouse LD_{50} titers of virus re
ered from blood, yolk sac, brain, and embryo body each 24 hours, from th
to the 18th day after inoculation The chart shows that virus, after
multiplication in the yolk sac, rapidly reached the blood stream, spread to
body, then to the brain, and reached peak concentration in these latter
tissues on the fourth and fifth days after inoculation. The virus conten
the yolk sac fell rapidly, starting on the fifth day and remained low thereaf
In the blood, virus diminished gradually, but considerable amounts could
be demonstrated up to the time of hatching and even in one-, two-, and th
day-old chicks The titers of virus in the body and brain dropped slowly a
the fourth and fifth days, with the concentration in the brain being consisten
higher than in the body, but virus was still present in both up to the time
hatching, and traces demonstrated in brain suspensions obtained from o
two-, and three-day-old chicks The MEF1 strain appears to multiply a
spread in the chick embryo in much the same way as the 17D strain of yell
fever virus was described by Fox and Laemmert[8] as doing in the same host

The virus distribution, as well as the pathological findings, suggested th
the stage of extraneural infection was followed by neural invasion with hi
titers of virus and specific poliomyelitis lesions in the nervous system.

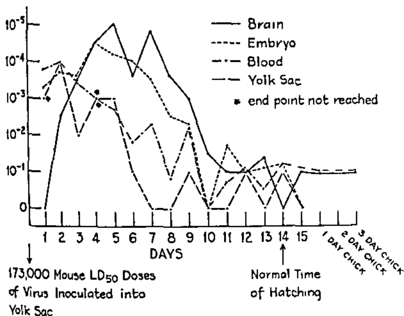


FIGURE 3 Daily distribution of virus in chick embryos infected with MEF_1 virus (series R [illegible] passage)

Studies were next undertaken to determine the pathogenicity of the chick-embryo adapted strain for primates, and to find out whether or not it possessed antigen- [illegible]

[illegible] impanzees received 2 0 ml of 20 per [illegible] 10,800 mouse LD_{50} doses of virus representing the 22nd egg passage (TABLE 1) Two animals were fed the inoculum, and two were injected intramuscularly The chimpanzees failed to

TABLE 1
RESULTS OF EXPOSURE OF CHIMPANZEES TO MEF_1 CHICK EMBRYO ADAPTED STRAIN (SERIES R 382 22ND PASSAGE)

Animal No	Route	First exposure		Second exposure	
		Dose	Neut index 30 days after exposure	Dose	Neut index 15 days after 2nd exposure
1	Oral	4 0	0	6 4	1 5
2	Oral	4 0	0	6 4	2 5
3	I M	4 0	1 8	5 6	2 8
4	I M	4 0	1 4	5 6	4 0

Dose and neut index expressed in mouse LD_{50} as log to base 10
No virus in blood 1 to 12 days after 1st exposure
No virus in stools 4 to 21 days after 1st and 2nd exposure

TABLE 2

DURATION OF IMMUNITY IN CHIMPANZEES EXPOSED TO MEF₁ CHICK EMBRYO ADAPTED STRAIN (SERIES R-382-22ND PASSAGE)

Animal No	Route	First exposure dose	Second exposure (1 month after 1st)				
			Dose	Neut indices after 2nd exposure (days)			
				30	45	60	180
1	Oral	4.0	6.4	1.3	1.3	2.0	1.9
2	Oral	4.0	6.4	1.8	1.5	2.4	1.3
3	I M	4.0	5.6	1.8	1.0	1.5	2.2
4	I M	4.0	5.6	4.0	3.5	3.1	2.5

Dose and neut index expressed in mouse LD_{50} as log to base 10
No virus in blood 1 to 12 days after 1st exposure
No virus in stools 4 to 21 days after 1st and 2nd exposures.

show any signs of illness during a 30-day observation period, and no virus was isolated from blood specimens taken 1 to 12 days after exposure. The animals exposed by the oral route did not develop antibodies, and those injected showed only an equivocal antibody reaction The four animals were re-exposed, with the same material and by the same route as before, but this time 2,200,000 mouse LD_{50} doses were fed and 440,460 injected The results are summarized in TABLES 1 and 2 Fifteen days after the second exposure, the sera of all animals contained antibodies, and the persistence of immunity is indicated by the neutralization indices of blood specimens taken 30, 45, 60, and 180 days after the second exposure No virus could be detected in stools collected from the 4th to the 21st day after the first and second exposures

Pathogenicity for Monkeys

Intracerebral inoculation The pathogenicity of the series R-382 strain of virus for monkeys was first tested by the intracerebral inoculation of 0 50 ml of 20 per cent chick embryo suspensions from the 32nd, 42nd, 62nd, 71st, 82nd, 93rd, and 104th passages into groups of four to six monkeys each (TABLE 3) One monkey alone out of 35 showed any clinical sign of poliomyelitis in this case, tremor Histopathological examination revealed spinal cord lesions in only eight of the animals, with the highest cervical or lumbar cellular loss 55 per cent in one monkey and 60 per cent in another

In addition to the above experiment, another was performed in which material from the 71st chick embryo passage was titrated intracerebrally in rhesus monkeys Four monkeys were injected per tenfold dilution of virus from 10^{-0} to 10^{-5} (20 per cent chick-embryo suspension was considered as undiluted material and had a mouse LD_{50} titer of 10^{-4}) None of the animals presented any poliomyelitis signs during one month of observation, and histological examination revealed no spinal cord lesions

Intraspinal inoculation. Eight cynomolgus monkeys were injected intraspinally with inocula from the 71st egg passage (TABLE 4) One-tenth ml of 20 per cent chick-embryo suspension, representing 33,320 to 86,600 mouse LD_{50}

TABLE 3

RESULTS OF INTRACEREBRAL INOCULATION OF MONKEYS WITH DIFFERENT PASSAGE LEVELS OF MEF₁ CHICK EMBRYO ADAPTED STRAIN (SERIES R 382)

C E Passage	Dose* injected	Ratio of monkeys with clinical signs	Ratio of monkeys with spinal cord lesions	Percentage of cellular loss in positive animals	
				Cervical	Lumbar
32	5.5	0/4	2/4	20	<10
				20	18
42	5 2	0/4	0/4		
62	5 5	0/4	0/4		
	4 8	0/6	1/6	20	20
				15	18
71	6 0	0/5†	3/5	32	60
				10	20
82	6 0	0/4	1/4	39	[illegible]
93	5.5	0/4	0 4		
104	6 5	1/4‡	1/4	34	[illegible]

* Mouse LD_{50} expressed as log to base 10
† Cynomolgus monkeys, others rhesus
‡ Tremors

TABLE 4

RESULTS OF INTRASPINAL INOCULATION OF CYNOMOLGUS MONKEYS WITH MEF₁ CHICK-EMBRYO ADAPTED STRAIN (SERIES R 382 71ST PASSAGE)

Dose*	Ratio of monkeys with clinical polio	Spinal cord lesion: Per cent cellular loss	
		Cervical	Lumbar
4 9	0/4	0	0
		0	0
		0	0
		0	0
4 5	0/4	0	0
		19	27
		0	0
		0	0

Inoculum 0 10 ml 20% C E suspension
* Expressed in mouse LD_{50} as log to base 10

loses of virus, was given each monkey No signs of illness were observed in any of the animals The cords of all the monkeys were examined histopathologically Cellular loss was found in the cord of only one monkey, and that was low

Inte... [illegible] intravenously [illegible] virus None [illegible] no evidence of [illegible] ...tory changes nor appreciable cellular loss was found [illegible] the examination of their spinal cords

Intramuscular inoculation In another test, 71st chick-embryo passage was injected intramuscularly three consecutive times into seven cynomolgus mon-

TABLE 5

RESULTS OF I M INOCULATION OF MEF₁ CHICK-EMBRYO ADAPTED STRAIN (SERIES R-382) INTO CYNOMOLGUS MONKEYS

Number of monkeys	1st Inoculation		2nd Inoculation		3rd Inoculation	
	Dose	Neut index* 21 days after inoc	Dose	Neut index* 15 days after 2nd inoc	Dose	Neut index* 21 days after 3rd inoc
3	6 2	2 1	6 3	2 5	6 3	3 0
4	6 2	2 7	6 3	3.2	6 3	3 4

Inoculum 5 0 ml 20% C.E suspension.
Dose and neut index expressed in mouse LD_{50} as log to base 10
* *Pooled sera*

keys (TABLE 5). The first dose represented 1,433,000 mouse LD_{50}, the second, 25 days later, 1,866,000, and the third, after an interval of 40 days, 1,966,000 None of the animals presented any signs of poliomyelitis during an observation period of 100 days, and specific antibodies were demonstrated in two pools of sera consisting of blood samples from three and four animals respectively Significant neutralization indices of $10^{2 \ 1}$ for the first pool and $10^{2 \ 7}$ for the second were obtained 21 days after the first inoculation, and the booster effect of the second and third inoculations was indicated by successive increases in neutralization indices

Oral inoculation At the same time that the 71st egg passage virus was injected intramuscularly into cynomolgus monkeys, it was also fed three consecutive times to 12 animals of the same species (TABLE 6) The first feeding consisted of 2,866,000 mouse LD_{50} doses of virus, the second of 3,732,000, and the third of 3,833,000 Four of the monkeys of this group were used as controls for possible virus excretion No virus was demonstrated by the intracerebral mouse inoculation of material from pools of daily stool samples collected between the 4th and 16th days after the first feeding None of the monkeys showed signs of illness during the 100-day observation period No satisfactory antibody reaction was demonstrable in pools of sera from four

TABLE 6

RESULTS OF ORAL INOCULATION OF MEF₁ CHICK-EMBRYO ADAPTED STRAIN (SERIES R-382) IN CYNOMOLGUS MONKEYS

No of animals	1st Feeding		2nd Feeding		3rd Feeding	
	Dose	Neut index* 21 days after feeding	Dose	Neut index* 21 days after feeding	Dose	Neut index* 21 days after feeding
4	6 5	1 0	6 6	0 3	6 6	0 6
4	6 5	0 7	6 6	0 9	6 6	1 2
4*	6 5	0 2	6 6	0 8	6 6	1 1

m samples

monkeys each obtained 21, 15, and 21 days after the first, second, and third feedings, respectively.

Behavior of the virus in tissue culture Serial passages in trypsinized monkey kidney tissues were established in order to determine whether the MEF1 chick embryo adapted strain maintained the general characteristics of the parent poliomyelitis virus, *i e*, the ability to grow and produce cytopathogenic effect in cultures of monkey renal epithelial cells. TABLE 7 summarizes the results of two experiments, for each of which the inoculum was chick embryo suspension from the 71st egg passage In the first, initiated with 133,200 mouse LD doses, the virus was maintained in serial passages up to the 23rd generation when it disappeared. In the second, with an inoculum of 25 320 mouse LD

TABLE 7

RESULTS OF SERIAL MONKEY KIDNEY TISSUE CULTURE PASSAGES OF THE MEF1 CHICK EMBRYO ADAPTED STRAIN (R 382-71ST EGG PASSAGE)

Passage No	Experiment 1			Experiment 2		
	Cytopathogenicity		Mouse LD_{50} titer*	Cytopathogenicity		Mouse LD_{50} titer*
	No tubes	Degree		No tubes	Degree	
1	4	0	3 1	4	0	2 6
2	4	0	3 0	4	0	2 6
3	4	0	1 6	4	0	2 5
4	4	0	3 0	4	0	2 5
5	4	0	2 5	2	++++	0 6
				1	+	
6	4	0	3 1	3	0	1 5
7	4	0	2 0	4	0	1 8
8	4	0	1 0	4	0	1 0
9	4	0	2 5	3	0	1 7
				1	+	
10	4	0	1 0	4	0	1 0
11†	2	++++	1 0	4	0	1 0
	3	0				
12†	1	+++	2 1	3	+	1 7
	3	0				
13	2	++	3 0	4	0	1 5
	2	+				
14	4	0	1 5	3	0	1 6
15	4	0	<1 0	3	0	1 2
16	4	0	<1 0	3	0	1 5
17	4	0	0 5	4	0	<0 5
18	4	0	<1 0	2	++++	0 5
				1	+	
				3	0	
19	2	+++	<0 5			
	2	++++				
20	4	++++	<0 5			
21	5	0	<0 5			
22	3	0	<0 5			
23	3	0	<0 5			

Inoculum for experiment 1—21,320 mouse LD_{50}
Inoculum for experiment 2—25,320 mouse LD_{50}
* Expressed as log to base 10
† Positive tubes only used for passage and mouse titration

doses, the virus was passed up to the 18th generation. Some cytopathogenic

10th subculture of the first series and a pool from the 11th and 12th passages of the second series were injected into eggs. In spite of the low concentration of virus inoculated—330 mouse LD_{50} doses in one instance and 1,630 in the other—the two series have now gone through 6 and 16 serial passages in eggs

Since the virus had showed some cytopathogenic effect in tissue culture, it was thought advisable to determine whether the agent might have regained virulence for primates. Two rhesus monkeys were injected intracerebrally with 0.5 ml of undiluted material containing 16,665 mouse LD_{50} doses of virus from the 13th passage of the first tissue culture series. Neither monkey showed clinical signs of illness, nor were lesions discovered in the pathological examination of their spinal cords

Attempts to establish serial monkey passages. Attempts were made to establish serial passages in rhesus and cynomolgus monkeys by the intracerebral or intraspinal inoculation of virus. These experiments were undertaken in order to determine whether the chick embryo adapted strain would increase in virulence or regain pathogenicity after several passages in primates

One attempt at intracerebral serial passage was performed in rhesus monkeys It was initiated with 133,200 mouse LD_{50} doses of virus per animal, using an inoculum of 0 5 ml of 20 per cent chick embryo suspension from the 71st egg passage Pairs of monkeys were used in each of three passages One animal was sacrificed seven days after inoculation, and its spinal cord made into a 20 per cent suspension which was passed into two additional monkeys The cord suspension was also titrated intracerebrally in mice One monkey of each pair was kept as control for one month of observation for clinical signs and histopathological study *A similar series of passages was performed* in pairs of cynomolgus monkeys In both experiments, the same negative results were obtained: no virus was demonstrated from the first passage nor from subsequent "blind" passages The control monkeys did not develop any clinical signs of poliomyelitis, nor were pathological lesions found in the cords

The establishment of intraspinal serial passages was tried in cynomolgus monkeys (FIGU ... of virus
was used from ... an
inoculum of 0 1 ... of
monkeys were injected, one in each pair serving as control, and the spinal cord of the other being made into the 20 per cent suspension for monkey passage and titration in mice This series was carried through five passages. From the ... 10^{-27}, 10^{-14},
It could
None
of the control monkeys showed any clinical signs of poliomyelitis The control monkey of the first passage presented mild spinal cord lesions The cellular loss was 27 per cent cervical and 28 per cent lumbar. The other control monkeys had no histological cord lesions

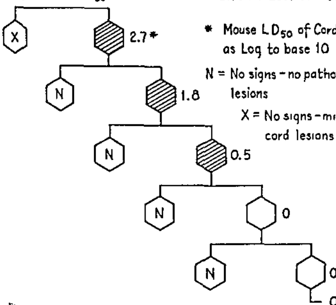


FIGURE 4 Attempted serial intraspinal passage of MEF1 chick embryo adapted strain in cynomolgus monkey (series R 382 71st passage)

The virus, after the monkey passages, retained its ability to grow in the chick embryo Serial egg passages were re-established with inocula of brain and spinal cords from mice infected with cord suspension from the third monkey "blind" passage, and from the monkey cord directly

Summary and Conclusions

Results of studies are presented indicating that a variant of the MEF_1 strain of poliomyelitis virus which has been propagated serially in the chick embryo has the following characteristics

(1) Modified pathogenicity for primates It induced a mild poliomyelitis attack in only one monkey out of 35 injected intracerebrally, and produced slight h...

... animals No poliomyelitis ... intraspinally, and only one ... muscular route to cynomol... ...ught about no poliomyelitis symptoms, but induced the development of specific antibodies Chimpanzees infected intramuscularly or orally did not develop intestinal carriage, nor did they show any poliomyelitis signs. Their sera, however, revealed specific antibodies The virus appeared to be avirulent for cynomolgus monkeys ex-

posed orally by repeated massive doses, and for animals of this species injected intravenously.

(2) Lack of ability to induce uniformly a cytopathogenic effect when grown in cultures of trypsinized monkey kidney tissue, and no ability to multiply properly in such cultures as compared with the parent strain

(3) No apparent tendency to regain virulence by serial intracerebral or intraspinal passages in monkeys

(4) Maintenance of its growth characteristics in the chick embryo, even when subinoculated from tissue culture or monkey passages

The above facts, showing the loss of virulence of the chick embryo-adapted strain for primates, the stability of the modified virus, and the retention of its immunogenic capacity for chimpanzees and cynomolgus monkeys, naturally suggests investigating the use of this variant as an immunizing agent for man

Acknowledgments

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Discussion of the Papers

Doctor Hilary Koprowski (*Lederle Laboratories Division, American Cyanamid Company, Pearl River, N Y*): The production of variants is usually approached in either of two ways The first method—that followed in the two preceding papers—is the orthodox procedure. The virus is introduced into a host not susceptible in the ordinary course of events, and the appearance of variants is awaited as the virus propagates in the new milieu Viral literature is filled with descriptions of the production of such variants Sometimes, the adaptation may be to a different organ or tissue rather than to a new host

The second approach, the intentional, is a contemporary one. It is an attempt to change the characteristics of a viral p[illegible] according to certain

TABLE 1
PATHOGENICITY FOR MONKEYS OF TWO SUBLINES OF TN STRAIN POLIOMYELITIS VIRUS

Passage	PD_{50}	Route	Paralytic ratio of monkeys at various dilutions of virus					
			10^{-1}	10^{-2}	10^{-3}	[illegible]	[illegible]	
35 CR	3 7*	I C	22/44					
3 CR + 47 TC†	4 2	I C	0/4	0/4	1 3	1 ;	0 3	0
27 mouse (PRI)	5 7	I C	0/4	0/4	0 4	0 4	0 4	
	4 7	I S	0/4	0/4	0 4	0 4		

CR = cotton rat, TC = tissue culture.
* At 10^{-1} dilution
† Eleven passages at limited dilutions
‡ Progeny test 4.2 PD_{50} into monkeys 0 8 P R

genetic principles known or assumed to be true. This is a demanding [illegible] controversial task The method has been applied most extensive[illegible] field of animal viruses—to influenza The next paper will discuss the [illegible] findings, using the second approach in relation to the poliomyelitis virus

To return to the first approach, however· Doctor Schaeffer has described experience with the adaptation of poliomyelitis virus to mice and Doctor Roca-García has reported what can happen in the chick embryo. It is also be of interest to mention briefly changes which have been observed in the character of a poliomyelitis virus strain in the course of its propagation in two different species of rodents The origin of the three sublines summarized in TABLE 1 are the same. After 35 passages in cotton rat brain, the virus was found to be pathogenic for monkeys injected intracerebrally The paralysis rate was 50 per cent, and half of the paralyzed monkeys died The same strain, after three cotton rat passages, was grown in tissue culture of monkey kidney epithelium for 47 generations (36 passages at low dilution and 11 at limiting dilution) Although the particles pathogenic for monkeys were apparently thinned out, two monkeys injected with higher dilutions of the virus became paralyzed

Growth of the same strain in PRI mice resulted in the production of a variant which, when injected into monkeys either by the intracerebral or intraspinal route, failed to elicit signs of paralysis The variant was also noncytopathogenic for either human or monkey epithelial cells or fibroblasts in tissue culture

A wide range of behavior has been observed as viruses have been introduced into new environments For instance, a strain of poliomyelitis virus propagated in chick-embryo tissue culture may retain cytopathogenic properties for monkey kidney epithelium and, at the same time, lose its ability to infect mice, even when injected intraspinally.

The number of variants probably grows in direct proportion to the number of laboratories working with poliomyelitis virus In spite of increased attempts at the direct genetic control of viral populations, the usual approach to the modification of a virus, particularly as far as virulence is concerned, is by propagation in a new host.

CHARACTERISTICS AND GENETIC POTENTIALITIES OF EXPERIMENTALLY PRODUCED AND NATURALLY OCCURRING VARIANTS OF POLIOMYELITIS VIRUS*

By Albert B. Sabin

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Recently accumulated data are in accord with the hypothesis that the vary-ing pathogenic and immunogenic activities of poliomyelitis viruses in differen hosts and by different routes involving different cell types in the same host ar determined by virus particles with distinct genetic characters It follow from this that different variants can arise by mutation, and that the compositio of any viral population would depend on the selective influences of the tissu and host employed for propagation. In order to make progress in this field, i is necessary to define clearly the characters exhibited by different poliomyeliti viruses and to study them in a quantitative manner. Characters having to d with virulence, immunogenic capacity, and cytopathogenic activity *in vit* readily lend themselves to such quantitative study. For poliomyelitis viruse virulence is synonymous with paralytogenic activity resulting from damage t large numbers of lower motor neurons The ultimate test for this characte is inoculation in the gray matter of the spinal cord. It is already known th the characters that determine virulence for newborn mice, adult mice, mon keys, and chimpanzees are distinct The position of hamsters and cotto rats has not as yet been clearly defined. Variants that are avirulent after in tracerebral inoculation, but virulent after spinal inoculation lack the charact that enables the virus to multiply sufficiently in certain areas of the brainste *to permit spread to the lower motor neurons* However, *when very lar* quantities of intracerebrally avirulent variants are inoculated into the sub space of a mouse's brain, or even a monkey's brain, a sufficient number of vir particles may reach the lower motor neurons directly without having to pa *through a barrier of insusceptible cells in the brainstem.* Thus, virus popul tions can be characterized with respect to intracerebral and spinal virulen only by quantitative tests and by progeny tests on the virus recovered from t paralyzed animal. It has also been established that there are different grad of virulence for both intracerebral and spinal routes, depending on the capaci of the variant to multiply sufficiently in the initially invaded cells to perm extension to large enough numbers of others Thus, the intracerebral avirulent type 1 Brunhilde strain segregated by Enders, Weller, and Robbin in human tissue culture, the type 2 MEF_1 strain that we segregated by t terminal dilution technique in suckling mice, and the naturally *occurring ty* 2 and type 3 strains recovered from the stools of healthy children by Ramo Alvarez and myself[2] all produced lesions of varying extent either only in t brainstem or in the brainstem and spinal cord of the intracerebrally inoculat *monkeys that remained clinically well* On the other hand, the intracerebra

* Aided by a grant from the National Foundation for Infantile Paralysis Inc., New York, N. Y.

avirulent Mahoney, YSK and Leon strains that we[3] segregated in cynomolgus kidney cultures produced no lesions in the intracerebrally inoculated monkeys. After spinal inoculation of experimentally produced or naturally occurring intracerebrally avirulent strains, we also found marked variation in paralytic capacity, some producing only limited or mild and transitory paralysis and others more severe and extensive involvement, some producing paralysis regularly with as little as 1000 TCD_{50} of virus, and others producing paralysis irregularly with 100 to 1000 times as much virus and not at all with the smaller [illegible] These observations are of importance for definitive study and the [illegible] understanding of the genetic mechanism determining the characters of virulence, as well as to discourage any premature simple concepts of one "gene" or other "genetic unit" *for one character*

Virulence after oral or parenteral extraneural administration of poliomyelitis virus has been shown to be [illegible]

[illegible]

paralysis, a variant was obtained that was just as virulent quantitatively [illegible] intracerebrally inoculated cynomolgus monkeys but had lost most of its [illegible] ity to produce paralysis by the oral route and also had a markedly reduced capacity for producing inapparent infection by this route (TABLE 1). The evidence that this resulted from selection of a variant with special [illegible] constitution and did not represent a "host-induced" variation is derived from the demonstration that two consecutive passages of this variant in the central nervous system (CNS) of cynomolgus or rhesus monkeys failed to restore its virulence or high infectivity by the oral route. Immunogenic capacity by alimentary and parenteral routes has been found to depend on characters that permit adequate multiplication in certain nonnervous cells *in vivo*. These characters have been found to be distinct from those that determine *in vitro* cytopathogenic activity on epithelial cells or fibroblasts, or the [illegible] *on the lower motor neurons or neurons in the brainstem*

TABLE 1
DISSOCIATION BETWEEN INTRACEREBRAL AND ALIMENTARY PATHOGENICITY OF YSK VIRUS

Dilution of virus fed to cynomolgus monkeys	Results obtained with virus propagated in indicated manner			
	Mouse 14 + cynomolgus brain 2		Mouse 33	
	Cynomolgus intracerebral titer = 10[illegible]		Cynomolgus intracerebral titer = 10[illegible]	
	Paralysis	Total infection	Paralysis	Total infection
10[illegible]	27/48—56%	96%	0/18—0	17%
10[illegible]	22/90—24%	60%	1/19—5%	17%
10[illegible]	5/37—14%	43%	1/18—6%	6%
10[illegible]	0/18—0	6%	—	—
10[illegible]	0/18—0	0	—	—

Monkeys with lesions in olfactory pathways excluded from this analysis

TABLE 2

SELECTIVE SUPPRESSION OF PARTICLES WITH ...
CYTOPATHOGENIC EFFECT ON MONKEY ...
MONKEYS OF TYPE 2 POLI...

Virus tested	Intracerebral titer $Log_{10}PD_{50}/gm$		Cytopathogenic titer $Log_{10}TCD_{50}/gm$	
	Mice	Cynomolgi	Cynomolgus kidney epithelium	Cynomolgus testis fibroblasts
Mouse passage 15	5 1	5 0	5 2	4 2
Mouse P 15 + cynomolg brain 2	2 8	—	5 4	0 or <1 0
Mouse P 15 + rhesus brain 2	<1 0	—	5 2	0 or <1 0
Mouse passage 30	4 2	—	6 2	5 2

Virus particles possessing certain sets of these characters can be selected for or against by propagation in different tissues of different hosts or by taking advantage of differential growth rates. The data presented in TABLE 2 show the selective suppression of particles with intracerebral virulence for mice and cytopathogenic effect on monkey fibroblasts by intracerebral passage of the type 2, YSK poliomyelitis virus in monkeys.[5] The mouse-passaged virus had practically the same number of infective units per gram of tissue as measured by intracereb...

cyt...

tite...

intr... passages in either cynomolgus or rhesus monkeys, the cytopathogenic activity for epithelial cells remained the same, while no cytopathogenic effect on fibroblasts was demonstrable. The intracerebral activity for mice was almost completely suppressed in the virus obtained from the rhesus monkeys and markedly reduced in that derived from the cynomolgus monkeys. Continued passage of this virus in mice yielded progeny with high activity on monkey fibroblasts.

Our earliest studies indicated that mere propagation in nonnervous tissue, such as cynomolgus kidney epithelium tissue cultures, does not lead to "host-induced," "nongenetic" variations in virulence of poliomyelitis viruses.[3,5] The type 1 Mahoney and type 2 YSK strains were maintained at full virulence for cynomolgus monkey by serial propagation in cynomolgus kidney epithelium, as long as minimal inocula were used and the culture fluids were not harvested until one or more days after complete cytopathogenic change was observed. This proved that the monkey kidney epithelial cells did not select against the f...

...cula

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...een

that, whenever a variant with a new set of characters appears as a result of a differential growth rate, the resulting viral population invariably consists of a mixture of the original and new types of particles. When the number of the new variant particles greatly exceeds the parent particles, it may be possible to segregate them by obtaining the progeny of individual or small numbers of

TABLE 3

TITRATION PATTERNS OF CULTURE FLUIDS CONSISTING OF DOMINANTLY VIRULENT, MIXTURE OF VIRULENT AND AVIRULENT, AND DOMINANTLY AVIRULENT VIRAL POPULATIONS BY INTRACEREBRAL TEST IN CYNOMOLGUS MONKEYS

Type 1 Poliomyelitis Virus—Mahoney Strain

Dilution of culture fluid inoculated (ml)	*Dominantly virulent kidney passage 10* Progeny of 9 terminal dilution passages from CNS $10^{?}$ TCD$_{50}$/ml	*Virulent + avirulent kidney passage 30* KP10 + 20 rapid passages with large inocula $10^{?}$ TCD$_{50}$/ml	*Dominantly [illegible]* Derived [illegible]
Undiluted	5/5	1/4	0/10
10^{-1}	5/5	2/4	0/4
10^{-2}	5/5	3/4	0/4
10^{-3}	5/5	2/4	0/4
10^{-4}	3/5	1/9	0/4
10^{-5}	4/5	0/4	0/4
10^{-6}	1/4	0/4	0/4
Severity of paralysis	100% Prostrate or dead Short incubation periods	13% Prostrate—others mild Long incubation periods	No para[illegible] No le[illegible]

virus particles in terminal dilution titrations. It has been demonstrated experimentally that virus particles with different sets of characters may interfere with one another[6] Accordingly, quantitative titrations are a better test for a given character than a test on the undiluted culture fluid In all such tests, one must have, as a standard of reference, the quantitative activity of the character that can best be measured in the medium or host in which the virus propagated, as well as the activity of the character under investigation The data presented in TABLE 3 show the intracerebral titration patterns in cynomolgus monkeys when culture fluids, consisting of dominantly virulent, a mixture of virulent and avirulent, and dominantly avirulent virus particles were tested[3] The results obtained with the kidney passage 10 culture fluid indicate that [illegible] effect [illegible]urthermore, the incubation periods were short, and prostrating paralytic or fatal infections were produced The kidney passage 30 culture fluid resulting from rapid passages with large inocula yielded a totally different pattern Most monkeys inoculated with 80 million TCD$_{50}$ developed neither paralysis nor lesions, but a larger number of the monkeys inoculated with less virus succumbed The monkeys inoculated with about 1,000 to 10,000 TCD$_{50}$ remained well The incubation periods were markedly prolonged in the succumbing monkeys, and the paralysis was generally mild and limited The evidence that these manifestations were due to a mixture of virulent and avirulent virus particles rather than to a predominantly homogeneous population of a new variant with diminished intracerebral virulence is based on two observations (1) recovery of fully virulent virus capable of producing prostrating paralysis after intracerebral inoculation of only 1000 TCD$_{50}$ from the spinal cord of one of the

TABLE 4
SPINAL VIRULENCE OF MONKEY INTRACEREBRAL-AVIRULENT VARIANTS IN MONKEYS AND CHIMPANZEES

No. of TCD₅₀ inoculated	Type 1 Mahoney, KP33			Type 2 YSK, KP51			Type 3 Leon, KP34		
Log₁₀	Cynomolgus	Rhesus	Chimpanzee	Cynomolgus	Rhesus	Chimpanzee	Cynomolgus	Rhesus	Chimpanzee
6 2–6 8	3/4	4/4	0/5	3/4	1/4	0/3	3/4	2/4	0/3
5 2–5 8	4/4	—	—	2/4	—	—	1/3	—	—
4 2–4 8	3/3	2/4	0/3	2/4	2/4	—	1/4	1/3	—
3 2–3 5	3/3	—	—	1/4	—	—	0/4	—	—
2 2–2 5	1/4	—	—	0/4	—	—	0/4	—	—
1 2–1 5	0/4	—	—	0/4	—	—	0/4	—	—
Extent of paralysis	Generally extensive	Generally slight or transitory	No paralysis, no lesions	Generally extensive	Generally slight or transitory	No paralysis, no lesions	Some extensive, some slight	Some extensive, some slight	No paralysis, no lesions

paralyzed monkeys (10^{-4} group); and (2) recovery of dominantly avirulent virus by the terminal dilution technique as shown in the results obtained with the kidney passage 33 culture fluid None of the 34 monkeys inoculated with 32 to 32 million TCD_{50} exhibited either paralysis or lesions Similar results were obtained with the type 2 YSK, and type 3 Leon strains[3]

The data shown in TABLE 4 indicate that the three monkey-intracerebrally avirulent variants just described possessed a varying degree of virulence for the lower motor neurons of cynomolgus and rhesus monkeys but not for those of chimpanzees[3,4] In spinally-inoculated cynomolgus monkeys the Mahoney strain was most active and the Leon strain least The rarity with which the type 3 variant produced paralysis with inocula of less than 10^6 TCD_{50} prompted a study to determine whether the culture fluid might contain less monkey spinal-virulent than spinal-avirulent particles The progeny of four separate terminal dilution tubes, however, exhibited the same limited spinal virulence in monkeys and it was concluded that the low incidence of paralysis was due to a limited capacity of all virus particles for multiplication and spread in the spinal cord All spinal inoculations were made into the gray matter of the lumbar cord and, when monkeys inoculated with any of the 3 types were sacrificed on the first day of paralysis, virus could be recovered from the lumbar cord but not from the cervical cord, and the amount of type 3, type 2 and occasionally, also type 1 virus was so low that spinal passage to another group of monkeys was not possible The type 1 and type 2 viruses were less active [illegible]

the eight chimpanzees inoculated with this strain shows that the character for spinal virulence is not the same for chimpanzees and monkeys

The data shown in TABLE 5 indicate that there is no relationship or linkage among the characters for cerebral and spinal virulence in mice and monkeys The parent Mahoney virus propagated in cynomolgus CNS lacked virulence for mice by either route, but the special kidney passage 33 derivative* possessed spinal virulence for mice but lacked cerebral virulence for monkeys[3] The parent YSK virus propagated in cynomolgus CNS was virulent for monkeys and mice by both routes, although the concentration of intracerebrally-mouse-virulent particles was at least 1/200 of the others The serial terminal dilution passages of this virus in cynomolgus kidney culture eliminated the mouse-intracerebral-virulent particles without affecting the others, while the special variant segregated in kidney passage 51 lacked cerebral virulence for monkeys and retained spinal virulence for mice and monkeys[3] The original Leon virus passaged in cynomolgus kidney culture possessed cerebral and spinal virulence for the monkey and only spinal virulence for the mouse, while the special variant segregated in kidney passage 34 lacked cerebral virulence for the monkey and spinal virulence for the mouse, but retained a limited degree of spinal virulence for the monkey.[3] To determine whether a few particles in the kidney passage 34 culture fluid might possess a special capacity for propagation in the

* The progeny from another terminal dilution tube of kidney passage 32 yielded a variant that was avirulent for mice by both routes and for monkeys by the intracerebral route

TABLE 5

LACK OF RELATIONSHIP AMONG CHARACTERS FOR CEREBRAL AND SPINAL VIRULENCE IN MICE AND MONKEYS

Poliomyelitis virus tested		Mice		Cynomolgus monkeys	
Type and strain	Line	Cerebral	Spinal	Cerebral	Spinal
1 Mahoney	Cynomolgus CNS	0	0	+	+
	Kidney passage 33 (Rapid passage + terminal dilution purification)—line 1	0	+	0	+
	—line 2	0	0	0	+
2 YSK	Cynomolgus CNS [illegible]	+	+	+	+
	[illegible]	0	+	+	+
	[illegible]	0	+	0	+
3 Leon	Testis 8, kidney passage 3	0	+	+	+
	Kidney passage 34 (Rapid passage + terminal dilution purification)	0	0	0	±
	KP34 + "blind" mouse spinal 1 + kidney passage 1	0	+	0	+

[illegible] stance, approximately 10- TCD$_{50}$ of virus was recovered per gram of spinal cord Although passage to other mice was negative, a progeny test on the virus that grew out in large amounts in cynomolgus kidney culture showed that a variant with new characters was segregated by this maneuver This variant possessed spinal virulence for mice and increased spinal virulence for monkeys, but was still intracerebrally avirulent for monkeys

Doctor Ramos-Alvarez and I studied the effect of rapid serial intraspinal passages in mice on spinal virulence for monkeys of the monkey-intracerebrally-avirulent Mahoney and YSK viruses Although this procedure enhanced the virulence for mice, no significant effect on monkey virulence was observed after 30 passages The mouse-adapted strain of Mahoney virus segregated by Li and Schaeffer[7] was made available to us for study after 35 consecutive spinal passages in mice The data shown in FIGURE 1 indicate that the cynomolgus spinal virulence of this strain was less than that of the Mahoney KP 33 which we had segregated in tissue culture Since only a portion of the monkeys developed paralysis, and since the affliction these monkeys showed was of a limited and mild type, it seemed desirable to determine whether this strain might represent a mixed population of monkey-spinal-virulent and spinal-avirulent virus particles When the undiluted culture fluids derived from 4 terminal dilution tubes were tested separately in a total of 24 cynomolgus monkeys, limited paralysis appeared in only 4 Higher dilutions of the same culture fluid, however, produced paralysis in a higher proportion of monkeys This zone phenomenon further suggested a "mixed-population" pattern and additional further terminal dilution purifications were carried out The 10^{-7}

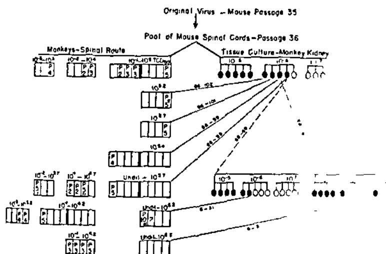


FIGURE 1. Spinal virulence in cynomolgus monkeys of Montgomery M

terminal dilution tube "6-51" is most likely to contain … infective particle captured in a single tube—most likely …

more rapid and extensive propagation in nonnervous tiss… effective as the parent viruses in their propagation in … One of the reasons the Mahoney strain was selected for spec… minimal amounts of it were capable of producing infection and immunity after extraneural, parenteral administration in monkeys. It was hoped that it might be possible to eliminate its high peripheral paralytogenic activity and retain its high peripheral infectivity. The data shown in TABLE 6 indicate that subcutaneous injection of 1 to 10 TCD_{50} of the original Mahoney virus sufficed to produce antib… well as paralysis. Although the variant con… passage 33 culture fluid no longer produced … contained in 0.01 ml. of culture fluid was re… cynomolgus monkeys. Since an occasional … (one millionth ml.), it would … certain cells and that much … results were obtained with … present data which in… viruses by the oral and … cynomolgus monkeys.

TABLE 5

LACK OF RELATIONSHIP AMONG CHARACTERS FOR CEREBRAL AND SPINAL VIRULENCE IN MICE AND MONKEYS

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Type and strain	Line	Cerebral	Spinal	Cerebral	Spinal
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	Kidney passage 33 (Rapid passage + terminal dilution purification)—line 1	0	+	0	+
	—line 2	0	0	0	+
2	[illegible] CNS	+	+	+	+
YSK	[illegible]	0	+	+	+
	[illegible]	0	+	0	+
3	[illegible]	0	+	+	+
Leon	[illegible]	0	0	0	±
	[illegible] sage 1	0	+	0	+

mouse spinal cord, 100 mice each received $10^{5.7}$ TCD_{50} of virus intraspinally Although no paralysis appeared, 20 mice were sacrificed at 14 days, and another 20 at 28 days and, in each instance, approximately $10^{2.7}$ TCD_{50} of virus was recovered *per gram of spinal cord* *Although passage to other mice* was negative, a progeny test on the virus that grew out in large amounts in cynomolgus kidney culture showed that a variant with new characters was segregated by this maneuver This variant possessed spinal virulence for mice and increased spinal virulence for monkeys, but was still intracerebrally avirulent for monkeys.

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TABLE 6
COMPARATIVE PARALYTOGENIC AND IMMUNOGENIC ACTIVITY IN INTRAMUSCULARLY I[N]LATED CYNOMOLGUS MONKEYS OF ORIGINAL TYPE 1 POLIOMYELITIS VIRUS (MAH[ONEY] STRAIN) AND VARIANT OBTAINED BY SPECIAL CULTIVATION IN CYNOMOLGUS KI[DNEY] TISSUE CULTURE

No of TCD_{50}* inoculated	Cynomolgus CNS suspension		Kidney passage 33	
	Paralysis	Antibody	Paralysis	Antibody
10,000,000	—	—	0/10	10/10
1,000,000	—	—	0/5	5/5
100,000	4/5	5/5	0/5	5/5
10,000	5/5	5/5	0/9	2/9
1,000	4/5	5/5	0/5	2/5
100	4/5	5/5	0/4	0/4
10	3/4	4/4	0/5	1/5
1	0/4	1/4	0/5	0/5

* TCD_{50} = 50 per cent tissue culture cytopathogenic dose

Since absence of intracerebral virulence for monkeys is one of the stri properties of the variants that appeared as a result of rapid passage with l inocula in monkey kidney cultures, and since such cultures do not select aga virus particles possessing the character for cerebral virulence except by di ential growth rates, it was desirable to study the effect of continued tissue ture passages on the genetic stability of the variants segregated by the term dilution technique. The test originally used consisted of inoculating gro of 10 monkeys with undiluted culture fluids that contained from 10^6 to TCD_{50} of virus. It was soon observed that in such tests one or more monl developed paralysis, and progeny tests revealed at least five different phen ena to account for it (TABLE 7) In the first phenomenon, paralysis app to be due to accidental invasion of cells susceptible to "spinal variant" parti and the progeny test yields virus that is cytopathogenic *in vitro*, but in cerebral passage in other monkeys is negative. In the second phenomen the progeny test yields *no* infective virus by any of the tests This app to be due to an abortive infection in which virus reproduction is initiated, ne cells are damaged, but the process of multiplication is not completed third phenomenon appears to be a variation of the second, in which a sr number of virus particles emerge that are strictly neurotropic. They do possess the *in vitro* cytopathogenic activity of the parent virus, but can p duce paralysis in monkeys that is transmissible to others with the product of virus that remains noncytopathogenic *in vitro* The second and third p nomena have been observed with the Leon strain, with the YSK strain, w naturally occurring type 2 spinal variant strains, and with the type 1 Br hilde, but never with the Mahoney strain The fourth phenomenon appe to be a mixture of the first and third phenomena. Here there is evidence roduction in the monkey spinal cord of two varieties of particles, some hich are noncytopathogenic and strictly neurotropic, while others are athogenic, spinal variants In the tissue culture test, there is evidence interference between the two in the form of long incubation periods, zo phenomena, and sparing of a portion of the cells On intracerebral passage

TABLE 7

PHENOMENA ENCOUNTERED IN PROGENY TEST FOR SIGNIFICANCE OF PARALYSIS IN OCCASIONAL MONKEY INOCULATED WITH LARGE AMOUNTS OF INTRACEREBRALLY AVIRULENT VIRUSES

Paralysis due to	Character of progeny in spinal cord of paralyzed monkey		
	Cytopathogenic effect in tissue culture	Intracerebral passage in monkeys	Intraspinal passage in monkeys
1 Accidental infection of cells susceptible to spinal variant	+ 10^5 to $10^7 TCD_{50}$/gm	O	+ or O Depending on titer of inoculum
2 "Abortive infection" No reproduction of infective virus "Sterile mutant"	O	O	O
3 Emergence of strict neurotropic mutant	O	Varying number paralyzed on 1st passage All paralyzed on 2nd passage Tissue culture test—Neg	Same as intracerebral
4 Mixture of 1 and 3	Long incubation Zone phenomena Portion of cells spared	Paralysis due to strict neurotropic variant	—
5 Emergence of particles with varying degrees of intracerebral virulence	+ Sometimes zone phenomena	+ Varying proportion of monkeys	—

TABLE 6

COMPARATIVE PARALYTOGENIC AND IMMUNOGENIC ACTIVITY IN INTRAMUSCULARLY INOCULATED CYNOMOLGUS MONKEYS OF ORIGINAL TYPE 1 POLIOMYELITIS VIRUS (MAHONEY STRAIN) AND VARIANT OBTAINED BY SPECIAL CULTIVATION IN CYNOMOLGUS KIDNEY TISSUE CULTURE

No. of TCD_{50}* inoculated	Cynomolgus CNS suspension		Kidney passage 33	
	Paralysis	Antibody	Paralysis	Antibody
10,000,000	—	—	0/10	10/10
1,000,000	—	—	0/5	5/5
100,000	4/5	5/5	0/5	5/5
10,000	5/5	5/5	0/9	2/9
1,000	4/5	5/5	0/5	2/5
100	4/5	5/5	0/4	0/4
10	3/4	4/4	0/5	1/5
1	0/4	1/4	0/5	0/5

* TCD_{50} = 50 per cent tissue culture cytopathogenic dose

Since absence of intracerebral virulence for monkeys is one of the striking properties of the variants that appeared as a result of rapid passage with large inocula in monkey kidney cultures, and since such cultures do not select against virus particles possessing the character for cerebral virulence except by differential growth rates, it was desirable to study the effect of continued tissue culture passages on the genetic stability of the variants segregated by the terminal dilution technique. The test originally used consisted of inoculating groups of 10 monkeys with undiluted culture fluids that contained from 10^5 to 10^8 [illegible] one or more monkeys

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s

to be due to accidental invasion of cells susceptible to "spinal variant" particles, and the progeny test yields virus that is cytopathogenic *in vitro*, but intracerebral passage in other monkeys is negative. In the second phenomenon, the progeny test yields *no* infective virus by any of the tests. This appears to be due to an abortive infection in which virus reproduction is initiated, nerve cells are damaged, but the process of multiplication is not completed. The third phenomenon appears to be a variation of the second, in which a small number of virus particles emerge that are strictly neurotropic. They do not possess the *in vitro* cytopathogenic activity of the parent virus, but can produce paralysis in monkeys that is transmissible to others with the production of virus that remains noncytopathogenic *in vitro*. The second and third phenomena have been observed with the Leon strain, with the YSK strain, with naturally occurring type 2 spinal variant strains, and with the type 1 Brunhilde, but never with the Mahoney strain. The fourth phenomenon appears to be a mixture of the first and third phenomena. Here there is evidence of reproduction in the monkey spinal cord of two varieties of particles, some of which are noncytopathogenic and strictly neurotropic, while others are cytopathogenic, spinal variants. In the tissue culture test, there is evidence of interference between the two in the form of long incubation periods, zone phenomena, and sparing of a portion of the cells. On intracerebral passage in

TABLE 7

PHENOMENA ENCOUNTERED IN PROGENY TEST FOR SIGNIFICANCE OF PARALYSIS IN OCCASIONAL MONKEY INOCULATED WITH LARGE AMOUNTS OF INTRACEREBRALLY AVIRULENT VIRUSES

Paralysis due to	Character of progeny in spinal cord of paralyzed monkey		
	Cytopathogenic effect in tissue culture	Intracerebral passage in monkeys	Intraspinal passage in monkeys
1 Accidental infection of cells susceptible to spinal variant	+ 10^{3} to 10^{7} TCD_{50}/gm	O	+ or O Depending on titer of inoculum
2 "Abortive infection" No reproduction of infective virus "Sterile mutant"	O	O	O
3 Emergence of strict neurotropic mutant	O	Varying number paralyzed on 1st passage All paralyzed on 2nd passage Tissue culture test—Neg	Same as intracerebral
4 Mixture of 1 and 3	Long incubation Zone phenomena Portion of cells spared	Paralysis due to strict neurotropic variant	—
5 Emergence of particles with varying degrees of intracerebral virulence	+ Sometimes zone phenomena	+ Varying proportion of monkeys	—

monkeys, a strict neurotropic variant is segregated. In the fifth phenomenon, there is evidence of selection of *in vitro* cytopathogenic particles with varying degrees of intracerebral virulence. On serial intracerebral passage in monkeys, one can segregate a population of virus particles that is intracerebrally virulent but that does not represent a reversion to the original parent virus because the characters for high extraneural infectivity and paralytogenic activity are missing.

For an adequate understanding of the tests for genetic stability on continued cultivation in tissue culture, it was deemed desirable to submit the starting stock cultures of each of the three type strains to still another test for homogeneity. The progeny of individual terminal dilution tubes were tested for intracerebral virulence in monkeys to determine whether the viral populations arising from single or small numbers of infective particles were uniform in relation to this character. FIGURE 2 shows the result of such a test on the Leon, kidney passage 34 stock culture fluid. When this fluid, which had a titer of $10^{5.4}$ TCD_{50} per ml, was tested undiluted and in tenfold dilutions to 10^{-4} in 31 intracerebrally inoculated monkeys, neither paralysis nor lesions appeared. Each oval in the diagram represents one monkey, and the figure over each group represents the number of TCD_{50} inoculated in each monkey. Negative re-

FIGURE 2 Test for homogeneity of intracerebral virulence in cynomolgus monkeys among progeny from indi-
vidual terminal dilution tubes (monkey kidney cultures) of Leon, KP 34 Leon KP 34 culture fluid tested undi-
luted and in tenfold dilutions to 10^{-4} produced neither paralysis nor lesions in 31 intracerebrally inoculated mon-
keys

sults in 10 monkeys were obtained with the progeny of 2 terminal dilution tubes. The test on the third terminal dilution tube, which contained more virus, was particularly illuminating since the five monkeys inoculated with $10^{6.3}$ TCD_{50} remained well, while three different phenomena were found to account for the poliomyelitic process which developed in three of the five monkeys inoculated with $10^{7.3}$ TCD_{50}. In the monkey with paralysis on the 19th day, the progeny yielded the pattern of "phenomenon 2," *i e*, abortive infection or incomplete reproduction of infective virus In the monkey with paralysis on the 17th day, the pattern corresponds to "phenomenon 4," with emergence of strict neurotropic virus particles In the monkey that died of intercurrent disease without paralysis but with poliomyelitic lesions, the pattern corresponds to "phenomenon 5," with emergence of intracerebrally virulent, *in vitro* cytopathogenic virus The zone phenomena observed in the tissue culture tests suggest that some noncytopathogenic, interfering virus particles were also being reproduced in the monkey CNS FIGURE 3 shows the results of the tests

FIGURE 3 Virus progeny tests on monkeys developing paralysis after intracerebral injection of culture fluids propagated with large inocula from Leon KP 34

for intracerebral virulence on some of the culture fluids from kidney passage 34 to kidney passage 60, as well as the progeny tests on the occasional monkey with paralysis. It is evident that intracerebrally virulent virus did not become dominant during the course of serial propagation in tissue culture. The progeny tests are similar to those just discussed, and they suggest that among each 10^6 to 10^7 infective particles there may be one that finds a selective medium when transferred to the monkey CNS, but not when it has to compete with a million or 10 million others in monkey kidney tissue culture. More extensive quantitative tests than those shown in this figure were carried out with the 60th passage culture fluid, both intracerebrally and intramuscularly, and no difference from the stock 34th passage culture fluid was found.

FIGURE 4 shows the homogeneity test for intracerebral virulence on the progeny of five different terminal dilution tubes of the YSK kidney passage 51 virus. There is no evidence here that the viral populations were different in the five tubes. Of the 24 inoculated monkeys, 4 developed poliomyelitis and the progeny tests again indicate that an occasional particle out of 10^6 to 10^7 may possess some capacity for propagation in the brainstem and thus be selected by intracerebral passage in the monkey. FIGURE 5 shows the results of intracerebral tests in monkeys on the stock YSK, KP 51 culture fluid

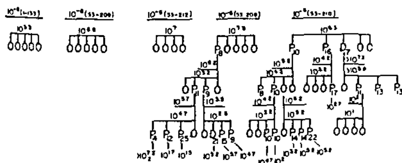


FIGURE 4. Test for homogeneity of intracerebral virulence in cynomolgus monkeys among progeny from individual terminal tubes (monkey kidney cultures) of YSK, KP 51. YSK, KP 51 culture fluid tested undiluted to 10^{-4} in 2 groups of 4 monkeys per tenfold dilution produced neither paralysis nor lesions after intracerebral injection.

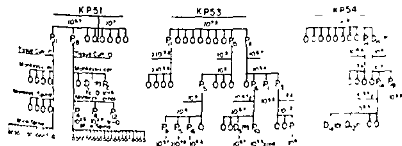
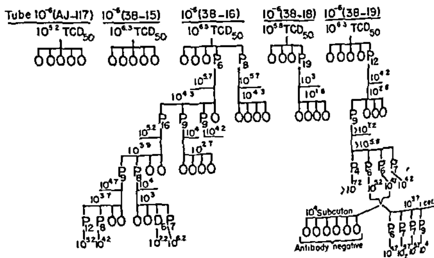


FIGURE 5. Progeny test on virus derived from cynomolgus monkeys developing paralysis after intracerebral injection of large doses of YSK, KP 51 and subsequent culture fluids propagated with large inocula.

and on other cultures derived from it by further passage The original KP 51 culture fluid with a titer of $10^{7.2}$ TCD_{50} produced neither paralysis nor lesions in any of 28 monkeys inoculated intracerebrally with tenfold dilutions from undiluted to 10^{-6}. When four additional monkeys were inoculated intracerebrally, however, paralysis appeared in two, and the progeny tests revealed the patterns of "phenomena 2 and 3" previously described It may be noted that in the monkey with paralysis on the 11th day, four different methods of testing for infective particles yielded negative results In the other monkey, a variant emerged that was neurotropic for the monkey but not for the mouse Among the 20 monkeys inoculated with 10^{7} to 10^{8} TCD_{50} of the KP 53 and KP 54 cultures, there were 5 with poliomyelitis The progeny tests on two of these showed that the paralysis was due to "phenomenon 1," *i e*, accidental invasion of cells susceptible to the spinal variant particles, since further intracerebral passage was negative. In the remaining three monkeys, the progeny test indicated the selection of particles with low grade intracerebral virulence More extensive quantitative tests for intracerebral and intramuscular paralytogenic activity on the 60th kidney passage culture fluid revealed no difference from the properties found in the stock KP 51 culture fluid.

FIGURE 6 shows the homogeneity test for intracerebral virulence on the progeny of five different terminal dilution tubes of the Mahoney kidney passage 33 virus There is also no evidence here that the viral populations were different in the five tubes Of the 24 inoculated monkeys, 4 developed poliomyelitis In 2 of these, the progeny test yielded the pattern of "phenomenon 1," *i e*, accidental invasion of lower motor neurons with negative intracerebral passage In the other 2, the pattern corresponds to the selection of particles



[illegible] monkeys among progeny from [illegible] of KP 33 culture fluid [illegible] rally in monkeys [illegible] 0/24 with [illegible]

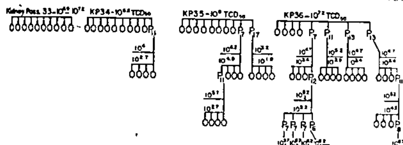


FIGURE 7 Intracerebral virulence in cynomolgus monkeys of progeny of modified Mahoney virus propagated with large inocula in 250 ml bottles

with varying degrees of intracerebral virulence In one instance, three serial passages failed to yield virus that was uniformly virulent by the intracerebral route while, in the other instance, two serial passages sufficed It is noteworthy that this does not represent a reversion to the parent virulent Mahoney strain, since the subcutaneous inoculation of 10^6 TCD_{50} in six monkeys not only failed to produce paralysis but also produced no antibody Similar negative results were obtained in tests on four additional intracerebrally virulent variants segregated from "Mahoney" culture fluids, indicating that the new mutants acquired the character for intracerebral virulence, but not the character for high extraneural paralytogenic activity The data shown in FIGURE 7 indicate that the Mahoney virus also could be passaged serially in monkey kidney culture fluid without change in its intracerebral virulence, except for the occasional presence of 1 particle in 10^7 to 10^8 which can be selectively segregated by intracerebral inoculation and passage in the CNS of monkeys

Finally, it is of interest to note that intracerebrally avirulent, spinal variant strains of poliomyelitis viruses are not merely laboratory artefacts but can also be recovered from healthy children who had no recent contact with clinically recognized cases of poliomyelitis[2] The data in TABLE 8 show tests on 4 such strains The type 2 strains were spinal variants for both monkeys and mice, while the type 3 strain, like the experimentally produced Leon variant,[3] was avirulent for mice by both routes

TABLE 8

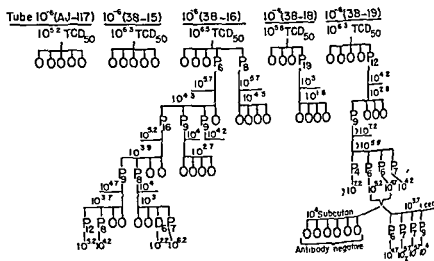
NATURALLY OCCURRING "SPINAL VARIANT" POLIOMYELITIS VIRUSES IN TISSUE CULTURES OF RECTAL SWABS FROM HEALTHY CHILDREN

Immunologic Type	Strain	TCD_{50} ml culture fluid Log_{10}	Effects in monkeys		Effect in mice	
			Intracerebral 0 5 ml	Intraspinal 0 1 ml	Intracerebral 0 03 ml	Intraspinal 0 02 ml
2	FAF-117	6 2	0/4	4/4	0/10	10/10
	FAF 116	6 7	1*/4	4/4	0 7	9/9
3	SAC 266	5 5	2†/5	4 4	0 10	5/10
	GLF	5 2	0/4	3/4	0/10	0/10

* "Sterile mutant" Tissue culture negative, intracerebral passage in monkeys negative
† Monkey 1—"sterile mutant" Tissue culture negative intracerebral passage in monkeys negative Monkey 2— spinal variant accident $10^{[illegible]}$ TCD_{50} gm with negative intracerebral passage in monkeys

and on other cultures derived from it by further passage. The original KP 51 culture fluid with a titer of $10^{7.2}$ TCD_{50} produced neither paralysis nor lesions in any of 28 monkeys inoculated intracerebrally with tenfold dilutions from undiluted to 10^{-6}. When four additional monkeys were inoculated intracerebrally, however, paralysis appeared in two, and the progeny tests revealed the patterns of "phenomena 2 and 3" previously described It may be noted that in the monkey with paralysis on the 11th day, four different methods of testing for infective particles yielded negative results In the other monkey, a variant emerged that was neurotropic for the monkey but not for the mouse. Among the 20 monkeys inoculated with 10^7 to 10^8 TCD_{50} of the KP 53 and KP 54 cultures, there were 5 with poliomyelitis. The progeny tests on two of these showed that the paralysis was due to "phenomenon 1," *i e.*, accidental invasion of cells susceptible to the spinal variant particles, since further intracerebral passage was negative. In the remaining three monkeys, the progeny test indicated the selection of particles with low grade intracerebral virulence More extensive quantitative tests for intracerebral and intramuscular paralytogenic activity on the 60th kidney passage culture fluid revealed no difference from the properties found in the stock KP 51 culture fluid

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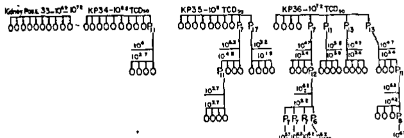


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3	GLE	5 2	0/4	3/4	0/10	0/10

* "Sterile mutant" Tissue culture negative intracerebral passage in monkeys negative
† Monkey 1— "sterile mutant" Tissue culture negative intracerebral passage in monkeys negative Monkey 2—"spinal variant accident" $10^{4.3}$ TCD_{50} gm with negative intracerebral passage in monkeys

Further work is now in progress on the recovery of strains that are completely avirulent for monkeys by the spinal route and, at the same time, more highly infective and immunogenic by extraneural routes Quantitative studies on the immunogenic and other properties of currently available chimpanzee-avirulent strains in chimpanzees are described by the author elsewhere in this monograph [8]

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Discussion of the Paper

DOCTOR JOHN F ENDERS (*Research Division of Infectious Diseases, Children's Medical Center, and Department of Bacteriology and Immunology, Harvard Medical School, Boston, Mass*): Doctor Sabin is to be congratulated on this important and extensive study of the capacity of poliomyelitis viruses to vary in their pathogenic properties after cultivation *in vitro*. The array of variants which he has thus produced differing widely in their cytopathogenic and paralytogenic properties will furnish materials for many future investigations For example, it will probably be desirable to define by the most accurate methods available the stability of the variants exhibiting the lowest pathogenicity for primates An approach to such investigations would seem to be provided by the establishment of "clones," using as primary inoculum, the virus produced by a single infected cell and obtained by the procedure described by Dulbecco elsewhere in this monograph The variants characterized by loss of cytopathogenicity *in vitro*, with and without the retention of the capacity to produce paralysis in susceptible animals, seem to provide exceptionally favorable materials for use in future attempts to discover those factors responsible for virulence or pathogenicity *in sensu strictu* as distinct from ability to multiply within the susceptible cell Experiments along such lines are much to be desired since, at the present time, we have no precise ideas in respect to the attributes that are lost or modified when variation in the direction of a decrease in pathogenicity occurs

Doctor Sabin's important findings are so numerous that it is impossible to discuss them in detail under these circumstances One specific comment, therefore, will have to suffice Doctor Sabin concluded that successive rapid passages *in vitro* and the use of large inocula were the principal factors responsible for the selection of variants exhibiting low virulence for primates While undoubtedly this conclusion is supported by the data presented, the possibility remains that other conditions may have been involved, since, as control, a like number of tissue culture passages carried out at longer intervals under otherwise comparable conditions were not affected In earlier experi-

ments of our own,* a considerable decline in the virulence for monkeys of the Brunhilde strain occurred during a series of passages that were done at intervals of two to three weeks in suspended cell cultures of human embryonic skin and muscle tissues Thus, although the experimental techniques were not entirely comparable, the possibility should perhaps be kept in mind that rapid passage *in vitro* may not represent an essential condition for the emergence of variants of low pathogenicity. That they regularly appear under these circumstances, has, however, been clearly demonstrated by Doctor Sabin's experiments

*Enders J F T H Weller, & F C Robbins 1952 Alteration in pathogenicity for monkeys of Brunhilde strain of poliomyelitis following cultivation in human tissues Federation Proc 11 467

SUMMARY AND REVIEW OF PAPERS ON "POLIOMYELITIS VIRUS VARIATION"*

By Hattie E. Alexander

The Babies Hospital and the Department of Pediatrics, College of Physicians and Surgeons, Columbia University, New York, N. Y.

The papers in this monograph dealing with "Variation" represent important contributions to the biology of poliomyelitis viruses. Mutation would seem to be a more appropriate term than variation when a variant trait can be shown to be heritable through many generations. The change can then be assumed to be a genetic one. The variant strains which have been described would appear to fullfil this criterion. This review will deal with mutation in the strict as well as the broader sense. Your chairman has asked me to say a word about our experience with "variants" of poliomyelitis.

The most complete knowledge of mutation of microorganisms has been derived from study of bacteria and bacterial viruses. In these microorganisms the heritable properties of a population can be changed either by (1) the selection and propagation of spontaneously occurring mutants, or (2) induction of genetic changes. In bacteria, genetic changes have been induced through transformation, transduction, and cell fusion and, in viruses, by recombination of parental genetic traits as they multiply in the same cell.

Selection of sponteneously occurring mutants. Bacteria and bacterial viruses have been the most valuable tools for demonstration of a principle of fundamental significance. Large populations can no longer be viewed as homogeneous with respect to those traits which are genetically controlled. The trait which has been studied most extensively for demonstration of this population heterogeneity is resistance of bacteria to antibiotics and bacterial viruses. In all species of bacteria examined, large populations sensitive to a given antibiotic can be shown to contain a minute number of cells that can grow in concentrations of the antibiotic that eliminate the masses. These resistant cells arise by mutation and, therefore, breed true. This principle applies to all bacterial species and all antibiotics examined.

The resistant trait is only one of many genetic changes which make it possible for bacterial mutants to survive in an environment which is lethal for the masses of the population. A bacterial population sensitive to a given phage, if large enough, will be protected from death of all cells by the presence of spontaneously occurring mutants which are resistant to the phage. On the other hand, a large phage population can find a host in which to propagate even in resistant bacterial populations through the appearance of mutant viruses that have genetically changed host requirements. This phenomenon might be viewed as a device to protect the race from extermination. The mechanism responsible for mutation, which we call spontaneous, is not known, but it appears to be controlled by some well-organized system. The rate of occurrence of a resistant mutant appears to be a constant in a given bacterial species against a given antibiotic or bacterial virus.

* The original work of the author on poliomyelitis viruses summarized in this review has been supported by the National Foundation for Infantile Paralysis, Inc., New York, N. Y.

The poliomyelitis strains described here exhibit heritable host requirements that differ from those of the parent populations. Each one could be viewed as a population evolving from spontaneously occurring mutants selected because they are equipped to reproduce in an environment which excludes the masses. Doctor Sabin has presented evidence on this point for his virus. The genetic change which increased the growth rate of Doctor Sabin's strain enabled it to overgrow the original population in the same environment. The differences in the characteristics of mutants selected from the same virus population demonstrate the heterogeneity of populations of poliomyelitis viruses just as has been shown for bacteria, bacterial viruses, influenza viruses, etc. Like other microorganisms, poliomyelitis populations cannot be viewed as being homogeneous with respect to a given trait, for example virulence or nonvirulence, for any length of time. The virulent one will contain avirulent particles and the nonvirulent population will show reverse mutation to virulence as described by Doctor Sabin for his strain. The evolution of these viruses has been studied in an environment in which the poliomyelitis population is the only microorganism present. One might anticipate that in the presence of other organisms, for example, in the intestinal tract, teeming with many varieties of microorganisms, there might be greater opportunities for change in virulence.

Induction of heritable changes in a population. Genetic changes have been induced in bacterial and bacterial virus populations by a variety of methods all of which provide an opportunity for interaction of the desoxyribose nucleic acids (DNA's) of two genetically different organisms. In each of these microorganisms, there is convincing evidence that DNA is the dynamic component of heredity determinants. The methods which provide an opportunity for interaction of DNA in bacteria are transformation, transduction, and fusion of cells.

Transformation. All evidence documents the fact that transformation induces a heritable change by exposure of the recipient cells to a specific chemical substance, DNA, isolated from appropriate donor cells. Type specificity and virulence have been induced by appropriate DNA's in pneumococci by Avery, McLeod, and McCarty and in *Hemophilus influenzae* and in meningococci in our laboratory.

By means of transformation, resistance to penicillin and streptomycin has been induced in sensitive populations of pneumococci by Hotchkiss, and resistance to streptomycin has been induced in sensitive strains of *H. influenzae* in our laboratory. It is of great significance that the pattern of degree of resistance induced is analogous to that which occurs naturally. A high degree of penicillin resistance is induced only by a stepwise process, whereas a high degree of resistance to streptomycin may be induced by a single step. It would seem that one can reproduce, by transformation, cells which cannot be distinguished from those that arise by spontaneous mutation.

Transduction. Lederberg and Zinder have demonstrated that new genetic traits can be induced in bacteria following invasion by appropriate bacterial viruses. The evidence suggests that genetic material can be carried by the virus from a previous host and incorporated in the genetic structure of in-

vaded bacteria. Transduction has changed the heritable properties of *Escherichia coli* and *Salmonella* and the induction of heritable virulence in avirulent *C. diphtheriae* is probably an example.

Fusion of cells. Tatum and Lederberg have shown that, when certain genotypically different strains of *E coli* are grown together, a small proportion of each of the populations seem to reproduce by a fusion process similar to sexual reproduction There is a recombination of the parental traits in the offspring and linkage of genetic traits along classic genetic lines

Recombination. The recombination method has been used primarily for changing the inherited properties of bacterial virus populations The results of interaction of the DNA's of two phages which infect the same cell are now well documented Recombination occurs The experimental evidence permits an interpretation of variation and evolution along more or less classic genetic lines

Of greatest interest is whether these principles that apply to bacteria and to bacterial viruses hold for animal viruses. Hirst, Burnet, and others have demonstrated recombination between two different antigenic types of influenza type A virus when they infect the same cell simultaneously under appropriate conditions

In our laboratory, Doctor Katherine Sprunt and Doctor Isabel Morgan Mountain have demonstrated an interesting variant that formed following the simultaneous infection of monkey kidney cells with type I and type II poliomyelitis viruses The presence of a combined form, showing antigenic traits of type I and type II viruses in the same particle, was identified by the method of Hirst. Since the combined trait has been transmitted through seven subcultures, five of which have been from limiting dilutions, there is a high probability that it is heritable The combined virus is unstable however, and would therefore, correspond to Hirst's X_2 influenza virus and not to the X_3 virus

Hirst's X_2 influenza virus and our combined poliomyelitis virus, both of which may be comparable to diploid heterozygotes, are similar in a number of respects to the new type of *H influenzae* cell, Sab, produced in our laboratory by the action of type A DNA on type B *H influenzae* Sab cells possess the antigenic traits of both type A and type B and reproduce in this form; they differ from type A and type B cells

It would seem that recombination can induce heritable changes in animal viruses, and there is ample evidence that by selecting out nonvirulent and other types of spontaneously recurring mutants, the heritable traits of a population can be changed Whether heritable changes can be induced by the other methods which are applicable to bacteria cannot be answered at present.

The similarities between bacterial transformation and virus invasion and reproduction within a cell merit a comment As pointed out by McCarty, Hotchkiss, and Horsfall, virus infection and bacterial transformation have, as common features, an invasive and replicative series of events Invasion of a susceptible cell by either agent, virus or DNA, is followed by a latent period during which the invading particle itself cannot be demonstrated Moreover, invasion or replication of the agent can be blocked by prior infection with related infectious agents

THE CHANGING AGE DISTRIBUTION OF PARALYTIC POLIOMYELITIS

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A number of studies on the age distribution of poliomyelitis have been made but, for the most part, they covered periods prior to 1945[1, 2, 3] In general the conclusion was reached that there had been a relative decrease in incidence of cases under five years of age, from about 1920 to recent years Only one of these has commented on the effect of including nonparalytic cases on age distribution

The present report is concerned primarily with changes in the age distribution of paralytic poliomyelitis Since reasonably complete information on different types of the disease, *i e*, paralytic and nonparalytic covering a sufficiently long period of time, and tabulated in detail by age groups was available from only one state, this paper will show, in considerable detail the trends which have occurred in Massachusetts since 1935 Data for the state of Maryland, however, were available for paralytic cases from 1925 to 1951 inclusive making it possible to determine trends in that area Data for shorter periods of time for other areas will be used to illustrate similar or divergent trends Since complete information is not available for all parts of the country it can not be assumed that changes that occurred in Massachusetts and Maryland have also occurred in all other sections of the United States

Percentage distribution of cases FIGURE 1 shows the percentage distribution of total cases by broad age groups over a period of four decades in Massachusetts and, for three recent 5-year periods, the distribution of paralytic and nonparalytic cases by the same age groups

The chart shows that, (1) there was a gradual decrease in proportion of cases under five years of age over the four-decade period, (2) there was an increasing proportion of cases in the 5- to 9-year and 10- to 19 year groups until 1935, when proportions became fairly constant from one 5-year period to another, and (3) a fairly constant proportion of cases reported in the ages over 20 years until 1940, when there was a sharp increase, especially in the 5-year period 1948 to 1952 The chart also shows that there has been a greater concentration of paralytic than of nonparalytic cases in the 0-to-4 and 20-and-over age groups since 1935, a date that marks the time when such data became available in Massachusetts On the other hand, in the 5-to-9 and 10-to-19 groups, there was a greater concentration of nonparalytic than of paralytic cases

FIGURE 2 shows the percentage distribution of cases reported in Massachusetts by single years of age under 20 The denominator used in calculating percentages was the total number of all ages It is presumed that most, if not all, cases in 1916 were paralytic, consequently a comparison of the distribution in 1916 with the distribution of paralytic cases in 1948 to 1952 would

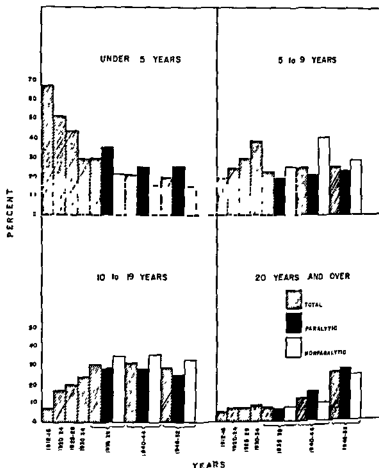


FIGURE 1 Per cent distribution of poliomyelitis cases by age and type of disease, Massachusetts, 1912 to 1952

be reasonably valid In 1916, the proportion of cases rose rapidly after the first year, reaching a peak in children two years of age The percentage of cases fell rapidly in subsequent years with a leveling off beginning at nine years of age. The cases reported in the 1948 to 1952 period have been separated into spinal paralytic, bulbar, and nonparalytic types (bulbar poliomyelitis and spinal paralytic cases were not tabulated separately until 1944). The same rapid increase in proportion of spinal paralytic cases after the first year, and reaching a peak in two-year old children, occurred in 1948 to 1952 The peak of cases was at a lower level, however, and the decline following the peak was more gradual than it was in 1916 Thus, the year of age with the greatest concentration of paralytic cases had not changed over a period of nearly 40 years, but there has been a more even distribution throughout the standard age classes in the 0-to-19 group.

The distribution of bulbar cases is markedly different from that of spinal paralytic. The percentage of cases under one year was less than one, and there

was a gradual rise in the proportion until a peak of 7.2 per cent was reached in 10-year-old children. The decline which followed was equally gradual. The distribution of nonparalytic cases was similar to that of bulbar cases, namely a gradual rise from a low point in the group under one year to those seven years of age and a gradual decline thereafter.

FIGURE 3 shows the percentage distribution of cases by single years in Minnesota in 1946. The distribution patterns of the various types are very similar to those for Massachusetts in 1948 to 1952.

The age distribution of cases by single years for New York City in 1944,

been shown separately, the distribution of spinal paralytic cases in New York City in 1944 would have been more nearly like that for Massachusetts and Minnesota.

The greater concentration of cases under five years in Southern states as compared with the distribution in the northern part of the country was pointed out many years ago. No data for recent years are available for Southern states in which there is a separation of paralytic and nonparalytic types of disease. The distribution of cases (paralytic and nonparalytic combined) however, would seem to indicate that there has been no change in the relationship between Northern and Southern states. For instance, the largest per

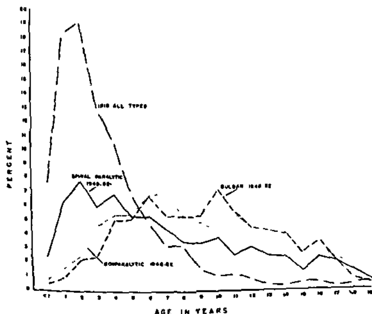


FIGURE 2 Percentage distribution of all poliomyelitis cases by single years of age under 20 Massachusetts All reported cases for 1916 by type, 1948 to 1952

centage of all reported cases (paralytic and nonparalytic combined) occurring in a single year in Massachusetts (1948 to 1952) was in those seven years old In Minnesota (1946), it was in the five-year-old cases In North Carolina (1948 to 1952) and Virginia (whites only—1949 to 1951) it was in cases three years of age. In Tennessee (whites—1948 to 1952), in two-year-old cases In Alabama (whites—1948 to 1952), in one-year-old cases

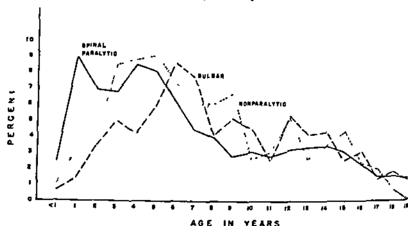


FIGURE 3 Percentage distribution of poliomyelitis cases by single years of age under 20 and by type, Minnesota, 1946

TABLE 1
POLIOMYELITIS MORBIDITY AND MORTALITY RATES BY AGE, AND RATIO OF RATE 5 TO 9 YEARS TO EACH AGE GROUP MASSACHUSETTS, 1916, 1935 TO 1939, 1940 TO 1944 AND 1948 TO 1952

Age	1916 Cases	1916 Deaths	Age	Total morbidity 1935–1939	Total morbidity 1940–1944	Total morbidity 1948–1952	Mortality 1935–1939	Mortality 1940–1944	Mortality 1948–1952
Average annual rates per 100,000 estimated population									
Under 1 year	185.8	66.9	Under 1 year	10.8	1.6	10.9	2.1	0	0.2
1–4 years	379.6	89.3	1–4 years	43.8	14.2	34.1	1.4	0.3	0.2
5–9 years	108.6	27.8	5–9 years	37.2	21.3	46.6	1.5	0.4	0.8
10–14 years	22.6	4.1	10–14 years	22.8	12.2	42.8	1.5	0.6	0.9
15–19 years	15.3		15–19 years	8.7	4.9	20.5	0.8	0.4	0.5
20–29 years	8.7	3.5	20–24 years	3.0	1.8	14.8	0.5	0.1	0.7
30–39 years	2.0	1.9	25–34 years	1.9	1.7	12.2	0.1	0.3	1.0
40–49 years	1.6	0.6	35–44 years	0.4	0.4	3.2	0.1	0.2	0.1
Ratio rate 5–9 to each age group									
Under 1 year	1.71	2.40	Under 1 year	0.29	0.07	0.23	1.40	—	0.25
1–4 years	3.49	3.21	1–4 years	1.17	0.66	0.73	0.93	0.75	0.25
5–9 years	1.00	1.00	5–9 years	1.00	1.00	1.00	1.00	1.00	1.00
10–14 years	0.21	0.15	10–14 years	0.61	0.57	0.92	1.00	1.50	1.12
15–19 years	0.14		15–19 years	0.23	0.23	0.44	0.53	1.00	0.62
20–29 years	0.08	0.12	20–24 years	0.08	0.08	0.32	0.33	0.25	0.87
30–39 years	0.02	0.07	25–34 years	0.05	0.08	0.26	0.06	0.75	1.25
40–49 years	0.01	0.02	35–44 years	0.01	0.02	0.07	0.06	0.50	0.12

Trends in morbidity rates by age No definite picture of the age distribution can be drawn, nor can conclusions be drawn regarding changes by comparing percentages for one period with another, because these data do not reflect differences in the age distribution of the population from one decade to another. Age specific morbidity or attack rates per 100,000 population must be used.

There has been a considerable amount of fluctuation in attack rates from one period to another in Massachusetts as shown in TABLE 1. These fluctuations, which are the result of relatively severe epidemics in some five-year periods and not in others, make it difficult to show a trend in rates of some age groups. This can be overcome to a great extent by using ratios of rates. Since the proportion of paralytic cases appears to have been more constant in the five-to-nine year group than in other ages, the ratio of the five-to-nine attack rate to that of each age group has been used to measure changes in age distribution of paralytic poliomyelitis.

In the upper part of TABLE 1, poliomyelitis morbidity or attack rates by age for all reported cases and mortality rates by age are shown for certain time periods. In the lower part of TABLE 1, the ratios of the five to nine rate to that of each age group are shown. In TABLE 2, the attack rates and ratios for each sex are tabulated for paralytic and nonparalytic cases in three recent five-year periods. The ratios for 1916 (all reported cases) and paralytic ...

TABLE 2

POLIOMYELITIS MORBIDITY RATES BY AGE, SEX, AND TYPE OF DISEASE AND RATIO OF RATE 5 TO 9 YEARS TO EACH AGE GROUP, MASSACHUSETTS, 1935 TO 1939, 1940 TO 1944, AND 1948 TO 1952

Age	Males						Females					
	Paralytic			Nonparalytic			Paralytic			Nonparalytic		
	1935–1939	1940–1944	1948–1952	1935–1939	1940–1944	1948–1952	1935–1939	1940–1944	1948–1952	1935–1939	1940–1944	1948–1952
	Annual average rates per 100 000 estimated population											
Under 1 year	7.8	1.3	9.3	2.1	0.6	1.9	10.9	0.7	7.6	0	0.7	2.9
1–4 years	52.7	13.2	27.0	13.0	4.3	12.2	24.9	8.4	19.0	8.5	1.9	9.8
5–9 years	22.2	12.8	26.9	20.8	12.4	30.3	18.8	9.0	19.8	10.8	6.7	15.4
10–14 years	14.7	8.0	27.0	12.2	6.1	28.3	10.2	4.3	15.3	7.0	4.9	14.6
15–19 years	6.1	2.9	10.9	4.3	1.7	13.8	3.7	2.8	9.7	2.8	1.0	6.6
20–24 years	1.2	1.2	7.3	1.4	0.4	8.5	1.9	1.2	7.8	1.4	0.5	5.7
25–34 years	1.4	1.3	6.6	0.7	0.2	5.1	1.1	1.3	8.3	0.6	0.6	4.5
35–44 years	0.3	0.5	1.5	0.3	0.1	1.1	0.3	0.1	1.9	0	0.2	1.4
	Ratio rate 5 to 9 to each age group											
Under 1 year	0.35	0.10	0.35	0.10	0.05	0.06	0.41	0.08	0.38		0.10	0.19
1–4 years	2.33	1.03	1.00	0.62	0.34	0.40	1.27	0.93	0.96	0.79	0.28	0.63
5–9 years	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
10–14 years	0.66	0.62	1.00	0.60	0.49	0.93	0.54	0.48	0.77	0.65	0.73	0.95
15–19 years	0.27	0.23	0.41	0.21	0.14	0.45	0.20	0.31	0.49	0.26	0.15	0.43
20–24 years	0.05	0.09	0.27	0.07	0.03	0.28	0.10	0.13	0.39	0.13	0.07	0.37
25–34 years	0.06	0.10	0.25	0.03	0.02	0.16	0.06	0.14	0.42	0.05	0.08	0.29
35–44 years	<0.01	0.04	0.06	0.01	0.01	0.03	0.01	0.01	0.09		0.03	0.09

tack rates for each sex for 1935 to 1939, 1940 to 1944, and 1948 to 1952 are plotted on FIGURE 4.

When the ratios for 1916 are plotted on a semilogarithmic scale, the result is a fairly straight line beginning with a relatively high morbidity under 5 years, as compared with that of the 5–9 group, and progressively lower rates in the age groups greater than 10.

When the ratios for 1935 to 1939 for each sex are compared with those for 1916, it becomes evident that a considerable reduction in morbidity had occurred under 5 years, relative to the 5-to-9 group. There was a relative increase, however, in rates in the 10-to-14 and 15-to-19 year groups for both sexes over the same period of time. In the age groups over 20 years, cases were too few in number to indicate significant trends, but there appeared to be no great amount of change.

The ratios for the 5-year period 1940 to 1944 indicated a continuing decline n morbidity under 5 years of age as compared with the 5-to-9 age group No

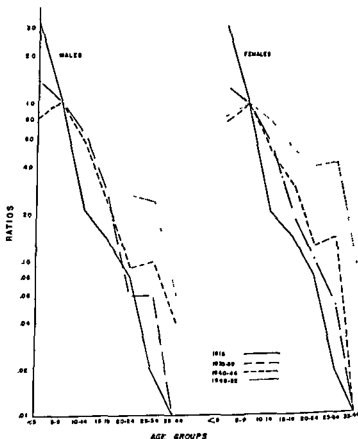


FIGURE 4 Ratio of paralytic poliomyelitis mor five to nine years to each age group, Massachusetts. Total cases 1916, by sex, 1935 to 1952

further change occurred in the 10 to 14 and 15 to 19 rates. In this 5-year period, there was a relative increase in morbidity, especially in females, in the 20 to 24, and 25 to 34 year groups.

There were different types of changes in the ratios in the 1948-to-1952 period. Under 5 years, the attack rates increased relative to the 5-to-9 group as compared with a decrease in the 1940-to-1944 period. Rates for the 10-to-14 and 15-to-19 age groups showed a moderate relative increase. A three-fold increase occurred in the age groups 20 to 24 and 25 to 34, as compared with the previous 5-year period.

The changes described above were also apparent in rates for nonparalytic disease as shown in TABLE 2. Nonparalytic rates under five years, however, were well below those for the five-to-nine group in each of the five-year periods.

Data on paralytic poliomyelitis for Maryland in the period from 1925 to 1951 inclusive are shown in TABLE 3. These figures indicate that the types of changes in age distribution in that state were similar to those found in the Massachusetts data, except that the magnitude of the increase in the rates for the 20-to-24 and 25-to-34 groups was less.

In Massachusetts, attack rates have been higher in males in all age groups throughout all of the periods covered in this report. The amount of increase in morbidity in ages over 20 from 1940 to 1944 to 1948 to 1952, however was greater in females. In Maryland, morbidity in females 20 years and over

TABLE 3

PARALYTIC POLIOMYELITIS MORBIDITY RATES PER 100,000 ESTIMATED POPULATION BY AGE AND SEX, AND RATIOS OF RATE 5 TO 9 YEARS TO EACH AGE GROUP. MARYLAND 1925 TO 1951

Age	Males					Females				
	1925–1929	1930–1934	1935–1939	1940–1944	1945–1951	1925–1929	1930–1934	1935–1939	1940–1944	1945–1951
Average annual rates										
Under 1 year	28.7	4.5	6.2	6.3	20.7	15.4	4.6	11.2	3.9	11.3
1-4 years	39.7	16.9	19.8	31.8	28.6	26.1	11.8	11.8	18.8	20.8
5-9 years	18.8	11.1	13.7	34.5	31.7	12.3	4.3	11.8	20.9	27.6
10-14 years	8.5	2.3	5.6	23.3	17.5	6.7	2.6	6.0	18.2	11.6
15-19 years	4.4	3.1	3.1	13.7	10.1	0.5	1.6	2.0	7.3	7.9
20-24 years	1.6	1.3	0.5	4.4	5.2	0.3	0.3	1.0	5.6	5.7
25-34 years	0.1	0.6	0.1	1.8	4.9	0.9	0	0.3	2.4	7.2
35-44 years	0.3	0.1	0.3	0.4	0.9	0	0	0	0.3	1.4
Ratios										
Under 1 year	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	1.07	0.95	0.18	0.48
1-4 years	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	1.00	0.90	0.75
5-9 years	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	1.00	1.00	1.00
10-14 years	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0.51	0.82	0.47
15-19 years	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0.17	0.35	0.28
20-24 years	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0.09	0.27	0.20
25-34 years	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	0.02	0.11	0.26
35-44 years	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	—	0.01	0.05

reached a higher level than in males in 1945 to 1951, although the amount of increase was less than in Massachusetts.

Even though paralytic cases could not be separated from the totals reported, age specific attack rates and ratios were calculated for total cases in North Carolina and white cases in Tennessee, but are not shown in tabular or graphic form. These ratios show that there had been a moderate decrease in the disease under 5 years relative to the 5-to-9 group in each state in the past 15 years. In Tennessee, there was a very slight increase in rates in ages 20 to 24 and 25 to 34 relative to the 5-to-9 rate and none in North Carolina. Whether or not paralytic rates in adults changed significantly in these states, as they did in Massachusetts and Maryland, could not be determined from the data that was available.

Mortality rates by age groups. Mortality rates have decreased to exceptionally low levels in Massachusetts in all age groups, especially under five years. Ratios of the rate five to nine years to each age group show changes similar to

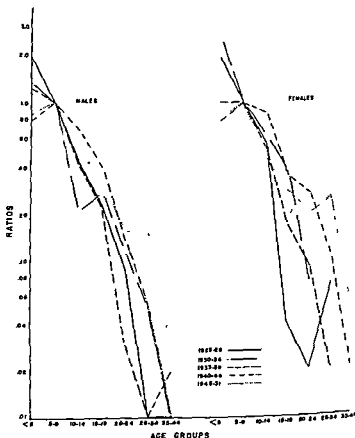


Figure 5. Ratio of paralytic poliomyelitis morbidity rate five to nine years to each age group by sex, Maryland, 1935 to 1951

those shown above for morbidity, except that rates under five years continued to decline in recent years. The sharp increase in mortality in young adults since 1940 parallels that of morbidity.

Discussion. The data presented in this paper confirm previous reports [illegible] incidence of paralytic poliomyelitis under five years has decreased when compared with rates for the five-to-nine age group. In Massachusetts [illegible] reversal of this trend in the 1948-to-1952 period, while in Mary[illegible] tinued, as shown in TABLE 3. It is apparent, however, that [illegible] rate for the age group one to four years has declined, it has [illegible] which is materially below that for the five-to-nine group [illegible] shown in FIGURES 6 and 7. In the five-year period 194[illegible] chusetts, the rates for males one to four years and five to [illegible] same and, for females, the one-to-four year rate almost equalled [illegible] In Maryland, the rates for males and females in the one-to-four [illegible] slightly below the five-to-nine rates for the years 1945 [illegible] available for California (1950 to 1952) and for New York [illegible] show that the attack rates in the one-to-four and five-to-nine [illegible] proximately equal in those areas.

The similarity in age distribution of bulbar and [illegible] Massachusetts was mentioned in a previous paragraph. [illegible] was also present in Minnesota and New York City [illegible] does not represent a peculiarity in reporting procedures. [illegible] similarity is not known, but the distribution of each type of disease probably depends on different types of factors. A causal relationship between [illegible]lectomy and bulbar disease has been established and since this operation is not often performed in early childhood, bulbar cases are infrequent under four years

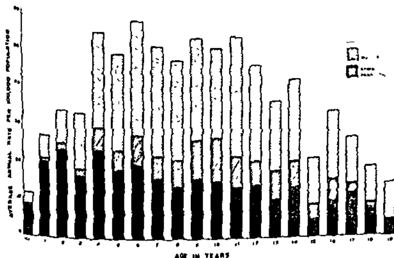


FIGURE 6. Poliomyelitis morbidity rates by single years of age and by type. Massachusetts, 1948 to 1952.

of age. The relative infrequency of reported cases of nonparalytic disease in early childhood might be due to nonrecognition, or because such types of cases are actually infrequent occurrences.

The prevailing view is that paralytic poliomyelitis is milder under five years than it is in subsequent age groups. As shown in TABLE 4, more cases per death were reported under five years than for other age groups in at least three states in recent years, a finding that supports such a statement. Bulbar cases, however, are less common as compared with other paralytic cases under five years of age. Since a fairly substantial proportion of bulbar cases terminate fatally, a higher percentage of these cases in the 5-to-9, 10-to-14, and 15-to-19 year groups may account in part for the greater "severity" of disease in the older age groups.

It is natural to look for reasons for the relative decrease of paralytic poliomyelitis under five years of age. One possible explanation is the falling birth rate and increasingly smaller families from 1915 to 1940. Since there was a reversal of the trend in attack rates under five years relative to those for the five-to-nine group in recent years which closely followed a marked increase in birth rates beginning about 1940, the falling birth rate as a factor in the relative decline in paralytic poliomyelitis under five years is given greater weight. It is not suggested, however, that the fall and subsequent rise in birth rates has been the only factor which has influenced trends in paralytic poliomyelitis under five years.

The explanation for the sudden rise in paralytic poliomyelitis in ages 20 to 24 and 25 to 34, which occurred in certain sections of the country in recent years, is not clear. Persons making up the age groups 20 to 24 and 25 to 34 in 1948

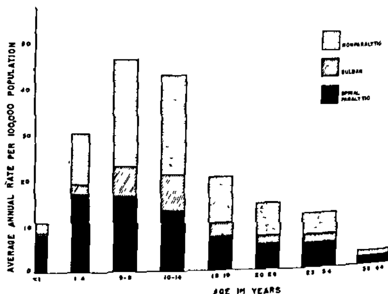


FIGURE 7 Poliomyelitis morbidity rates by age and by type, Massachusetts, 1948 to 1952

TABLE 4
NUMBER OF PARALYTIC CASES REPORTED PER DEATH

Age	Massachusetts 1948-1952	Minnesota 1946	Maryland 1948-[illegible]
Under 1 year	36	0	17
1-4 years	119	30	[illegible]
5-9 years	29	14	23
10-14 years	24	9	[illegible]
15-19 years	23	9	[illegible]
20-24 years	10	7	14
25-34 years	7	5	7
35-44 years	16	4	4

TABLE 5
RATIO OF BULBAR TO OTHER PARALYTIC CA[illegible]

Age	Massachusetts 1948-1952	Minnesota 1946	[illegible]
Under 1 year	1:17 0	1 11 0	[illegible]
1-4 years	1 7 0	1 7 6	[illegible]
5-9 years	1·2 5	1 2 9	[illegible]
10-14 years	1 1 6	1 2 6	[illegible]
15-19 years	1·2 8	1 3 4	[illegible]
20-24 years	1:3 1	1 2 4	[illegible]
25-34 years	1 3 1	1 2 2	[illegible]
35-44 years	1.5 2	1 2 2	[illegible]

to 1952 were for the most part born after 1916, i e , from about 1917 to 1932 This was a time when poliomyelitis morbidity was relatively low as compared with t[illegible]
culmi[illegible]
must [illegible]
years The persons born between 1917 and 1932 were the fathers and mothers [illegible]

[illegible] adults in the 1948 to 1952 period resulted, at least in part, [illegible] exposure to a child population in which incidence rates were high and on the increase It is also possible that introduction or dominance of new strains of virus in recent years, against which young adults had little immunity, could have played a part in higher attacks in these groups.

The poliomyelitis attack rates in the United States Army for the years 1939 to 1953 inclusive indicate a rise at the same time that rates began to increase in the civilian population. The average annual attack rate was 2 6 per 100,000 mean strength for troops stationed in continental United States for the years 1940 to 1944, as compared with a rate of 9.5 for the years 1948 to 1952 For personnel stationed outside continental United States, the rates were 3 6 and 12.3 respectively, for the same periods

The increase in attack rates in army personnel occurred at approximately the same time as the increase in young adults in Massachusetts Since the median age varied from 21 to 23 from 1946 to 1952, this would mean that many of the military having poliomyelitis in 1948 to 1952 were born between 1924 and 1930 inclusive. This corresponds with the period of relatively low morbidity in the United States

The data used in this report do not support the hypothesis commonly expressed by some investigators in attempting to explain the wide differences in age distribution and total incidence of poliomyelitis found in countries with high standards of living, as compared with areas where living standards are low These investigators have assumed that a larger proportion of persons living in countries where sanitary standards are relatively high escape infection early in life because of these superior standards Such postponement is said to account for high incidence rates in school children and in young adults It appears to the author that a more important factor in producing the changes in age distribution of the disease from 1916 to 1945 was the falling birth rate over these years—at least in Massachusetts. There is no evidence to indicate that the reversal in the trend of paralytic poliomyelitis under five years since 1945 [illegible] related to a lowering of sanitary standards in that state Changes in [illegible] other intrinsic and extrinsic factors need to be studied in much greater detail before assuming that standards of living alone will account for a large part of the differences in morbidity patterns found in different parts of the world

More information is also needed before trends in age distribution of poliomyelitis can be determined for various sections of the United States Retrospective studies covering states in each section, however, do not seem feasible [illegible] of cases [illegible] and for Southern as well as Northern states

Summary In Massachusetts, there has been a decline in percentage of poliomyelitis cases under five years of age in the four-decade period ended in 1952 Early in this period, the proportions rose in the 5-to-9 and 10-to-19 year age groups In recent years, there has been a sharp increase in cases 20 years of age and over

[illegible] by single years of age in Massachusetts [illegible] under five years with a peaking [illegible] types of disease are uncommon under 4 years of age and *the largest number occurred in* 10-year old cases Nonparalytic cases were most numerous in the 5-to-9 and 10-to-14 groups

Paralytic attack rates under five years have declined relative to the rates 5 to 9 years in both Massachusetts and Maryland *In 1948 to 1952*, however, there was a slight reversal in this trend

Since 1940, there has been a relative increase in attack rates in persons 20 years of age and over

It is suggested that the decline in paralytic poliom[illegible] five years was

related to declining birth rates, and the recent reversal of the trend in m... bidity was related to the increase in birth rates which began about 1940

The suggestion is made that the marked increase in attack rates over 20 occurred because this was a group that was relatively sus... the one hand, and was exposed to a young population with ... disease rates on the other

Acknowledgment This study would not have been po... operation of the following people and their staffs ... Doctor Roy Teemster, Massachusetts State Department ... H Mass, Doctor Gaylord Anderson, University of Minne... M... Minn, Doctor Morris Greenberg, New York City Depart... H... York, N Y; and Doctor Ralph Paffenbarger, Nation... biology, Bethesda, Md Data for the United States ... through the courtesy of E L Hamilton, Chief of the M... ision, Office of the Surgeon General, United States D... Washington D C Their assistance is grateful... ackno...

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POLIOMYELITIS: SEVERITY AND TYPE OF DISEASE IN DIFFERENT AGE GROUPS*

By Dorothy M. Horstmann

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Because of the changing age incidence of poliomyelitis in certain parts of the world, considerable attention has been drawn recently to differences in host responses associated with age The problems involved are of concern both to the epidemiologist and to the clinician, and they have practical implications in both fields.

That age *per se*, in addition to other host factors, such as genetic constitution, plays an important role in natural and experimental virus infections in animals is well recognized Theiler's virus, the Coxsackie group, various other neurotropic viruses, and the tumor viruses have been studied experimentally in this respect Marked differences in susceptibility to infection by different routes and, in some instances, marked differences in the character of the response have been observed.[1]

[illegible] in the human population is com-

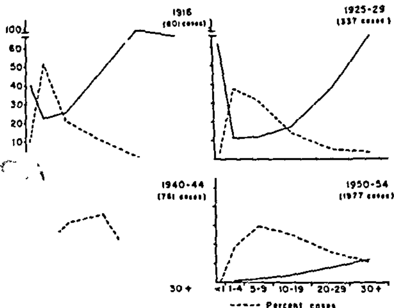
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in defining and reporting of nonparalytic cases About one aspect, namely severity, there is perhaps less confusion, at least if one considers mortality statistics as an index. In Connecticut, since 1916, the shape of the curve for case fatality per cent by age group has been relatively constant, with the lowest per cent of deaths in young children (except those under one year), the incidence rising [illegible] *with each age group* (FIGURE 1).† *This pattern has been main*taine[illegible], as elsewhere, despite marked shifts in age incidence of [illegible] a marked reduction in case fatality rates The higher mor[illegible] in 1925 to 1929 is explained in part by the fact that in, general, [illegible]ic cases were reported during this time, the practice of reporting [illegible]ic *cases not having become widespread until the 1930's*

[illegible] arises: Is the greater mortality in older age groups due to age or to some extrinsic factor or factors? This query cannot be answered [illegible] finality There is, however, some evidence to suggest that the greater [illegible]ity—and therefore mortality—in older age groups is perhaps enhanced by the *ill effects of exercise or continued activity, often carried out* under stress, *after the onset of symptoms of the major illness phase of the clinical disease* This is a far more common occurrence in adults than in children[2,3] Another contributing factor to the higher mortality in adults is probably associated with the relatively high incidence of the bulbar form of the disease in older age *groups in some outbreaks This is, however, a variable occurrence* and, in large series, the incidence of bulbar cases has not differed markedly in

[illegible] for Infantile [illegible]

Distribution according to age, and case fatality rates, Connecticut



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Bureau of Preventable Diseases, Connecticut State De

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POLIOMYELITIS: SEVERITY AND TYPE OF DISEASE IN *DIFFERENT AGE GROUPS**

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Because of the changing age incidence of poliomyelitis in certain parts of the world, considerable attention has been drawn recently to differences in host responses associated with age. The problems involved are of concern both to the epidemiologist and to the clinician, and they have practical implications in both fields.

That age *per se*, in addition to other host factors, such as genetic constitution, plays an important role in natural and experimental virus infections in animals is well recognized. Theiler's virus, the Coxsackie group, various other neurotropic viruses, and the tumor viruses have been studied experimentally in this respect. Marked differences in susceptibility to infection by different routes and, in some instances, marked differences in the character of the response have been observed.[1]

The age problem in relation to poliomyelitis in the human population is complicated by several factors, including the prevalence of mild or inapparent infection which cannot be recognized clinically, and the difficulties encountered in defining and reporting of nonparalytic cases. About one aspect, namely severity, there is perhaps less confusion, at least if one considers mortality statistics as an index. In Connecticut, since 1916, the shape of the curve for case fatality per cent by age group has been relatively constant, with the lowest per cent of deaths in young children (except those under one year), the incidence [illegible] has been main-

[illegible]

tality in 1916 and in 1925 to 1929 is explained in part by the fact that in, general, only paralytic cases were reported during this time, the practice of reporting nonparalytic cases not having become widespread until the 1930's.

The question arises. Is the greater mortality in older age groups due to age alone, or to some extrinsic factor or factors? This query cannot be answered with finality. There is, however, some evidence to suggest that the greater severity—and therefore mortality—in older age groups is perhaps enhanced by the ill effects of exercise or continued activity, often carried out under stress, after the onset of symptoms of the major illness phase of the clinical disease. This is a far more common occurrence in adults than in children.[2,3] Another [illegible] and with

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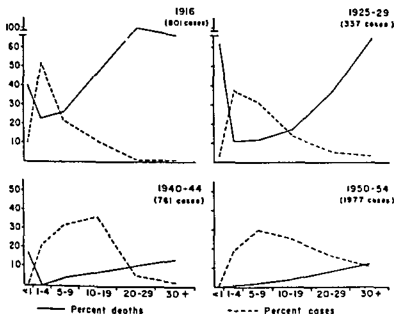


FIGURE 1 Distribution of cases according to age, and per cent of deaths by age group in Connecticut 1916 to 1954 (Data from Doctor James C Hart, Director, Bureau of Preventable Diseases Connecticut State Department of Health, Hartford Conn)

the various age groups Furthermore, high fatality rates in adults have been characteristic of epidemics in which bulbar cases have been almost absent

Epidemiological Aspects

Other aspects of the problem of age and severity of disease are contained in age specific attack rates for poliomyelitis in various parts of the world In order to interpret the data, some knowledge of the immune status of the population under study is essential Thus, in the native population of Casablanca, French Morocco, the attack rates for paralytic poliomyelitis are virtually nil for individuals over two years of age, and are low for the zero-to-two-year-age group. An explanation for the lack of cases in older age groups is apparent when data on the antibody patterns of the population are reviewed In a recent serological survey of this population,[4] we found that, after an initial fall in maternal neutralizing antibodies in the first few months of life, a rise in antibodies begins in the 7-to-11-month period, so that 75 per cent of children 12 to 23 months of age have neutralizing antibodies to at least one of the three types, and by 5 to 9 years of age, 80 per cent have antibodies for all three types of poliomyelitis virus' The higher attack rate in the under-two-age group is thus a reflection of the susceptibility of this age group, which invites a high infection

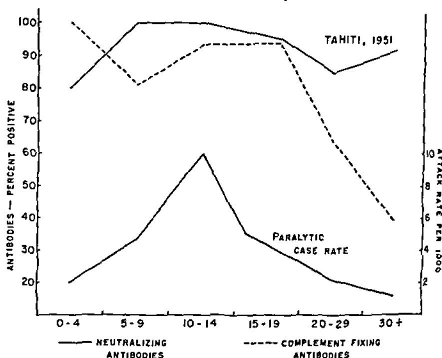


Figure 2 *Age distribution of antibodies to type 1 virus compared with age distribution of paralytic cases in the Tahiti epidemic, 1951* Sera, collected 6 weeks after the epidemic, were tested in 1 5 dilution in both neutralization and CF antibody tests (Data on attack rates from Rosen and Thooris [11])

months, 28 of the 29 paralytic cases on the other islands occurred in individuals over the age of 15 years

I have concentrated on these special outbreaks, which seem to illustrate the effect of age on the incidence of the paralytic disease, because they represent less complicated situations than elsewhere, and it has been possible to single out age and assume, with some slight degree of confidence, that other things were approximately equal. I hasten to add, however, that, in numerous outbreaks, the age pattern is confusing and confounding A good example is the outbreak which occurred in Jamaica in 1954* This was the first epidemic ever [illegible] groups had been

which I am familiar The explanation is indeed obscure.

The recent recurrent epidemics in Israel, beginning in 1950, also present some unusual features with regard to age distribution [19, 20] As with the earliest epidemics in other countries, the infantile group has been chiefly involved, 85 to 90 per cent of the cases being in children under four years old The extremely high attack rate which was found in the zero-to-four age group (93 per 10,000[20]) is probably associated with the practice of housing children together

* I am indebted to Doctor John R Paul, Yale University School of Medicine, New Haven, Conn, for information about this outbreak

in communal groups from birth. The spread of infection is thus comparable to its spread through enormously large families. However, unlike the New York 1916 epidemic and similar ones in which attack rates in the under-one year-olds have been half or less of the rates in one-to-four-year olds, in Israel there has been a consistently high attack rate in infants under one year. Of the 405 cases in this age group in 1950, 98 were between 0 and 5 months of age, and 307 between 6 and 11 months. This latter group actually had the highest attack rate of any age group, 189 per 10,000.[20] The usual situation, with relative sparing of infants under one year, has been attributed to the presence of passively transferred maternal antibodies. The lower incidence in Israel in the first 5 months than in the 6-to-11-month period would fall in with this view, although the occurrence of 98 cases in the 0-to-5-months group nevertheless represents an extraordinarily high attack rate. Since there is reason to suppose that these infants were probably born of mothers who possessed antibodies, the data suggest that protection from passively transferred antibody in the first year of life is a relative thing, and can be overcome in certain situations in which, for instance, a high dosage of a virulent strain is encountered. The same would seem to be true of immunity conferred by mothers' milk, which Sabin has found to be correlated with the presence of maternal serum antibody.[11] Of the Israel cases of 1950 in infants under one year on whom a feeding history was obtained, 85 per cent were breast-fed.[20]

In summarizing, then, the discussion so far on the effect of age on the severity and prevalence of poliomyelitis:

First, severity as measured by *mortality* figures indicate a high mortality in the first year (particularly in the first six months) of life, a situation common to [illegible] oup. The lowest mortality for [illegible] to four years. Increasingly [illegible]

[illegible] t one in a relatively isolated [illegible] young [illegible] nd in- [illegible] with severe paralysis and death occur much more commonly in young adults, whereas young children are more apt to have mild or inapparent infections.

Third, in many epidemics in which the population is partially immune, so many unknowns and variables present themselves that the effect of age *per se* cannot be assessed with any confidence.

Clinical Aspects

[illegible] certain [illegible] clin [illegible] the [illegible] by our department of three epidemics in 1948,[21] about 400 patients were interviewed by one person and complete histories taken either from the patient or, in the case of young children, from the parents. As a result of analysis of this material, several differences in clinical pattern were noted. These are summarized in TABLE 2. The biphasic "dromedary" course was noted in approxi-

Table 2

Comparison of Symptomatology, Children and Adults

Type of disease	Clinical characteristic	Age 2 to 9		Age 15 and ov	
		Number	Per cent	Number	Perc
Paralytic and non-paralytic	Biphasic course	69/188	37	13/117	11
	Major illness—				
	sudden onset	156/188	82	47/117	40
	gradual onset	32/188	18	70/177	60
	Pain—day 1 of major illness	91/188	43	89/177	76
Paralytic	Very severe back pain (relieved by motion)	4/117	2	27/90	30
Paralytic	Prodromal symptoms preceding major illness				
	incidence	19/119	16	34/94	36
	pain, any type	9/119	8	24/94	26
	hyperesthesia or paresthesia	1/119	1	10/94	11

mately 37 per cent of 188 children aged 2 to 9, but in only 11 per cent of 11 individuals 15 and over. The incidence of a biphasic course bore no relation to the type of disease, *i e*, paralytic or nonparalytic. The mode of onset of the "second" central-nervous-system phase, or "major illness" as we prefer to call it, since it occurs more often without a previous first phase, also differed in the two groups The onset was sudden with definite and striking fever and other symptoms and signs in 82 per cent of children, but in only 40 per cent of adults In contrast, 60 per cent of adults had a gradual, relatively afebrile onset, often preceded by a prodromal period of several days to a week or more During this time, pain, hyperesthesia, and paresthesia contributed to produce a rather bizarre clinical picture. Pain, as a presenting symptom, was more commonly encountered in adults than in children It was a first-day complaint in 76 per cent of adults, as com
course, pain was a pı
type of severe pain—
by motion—was encountered but rarely in children who developed the paralytic disease, but was seen in one third of paralytic cases over 15 years Pain of this type was not encountered in nonparalytic cases, and 21 of the 27 cases over 15 in which it occurred subsequently developed severe paralyses Thus, this symptom may be considered to have some prognostic significance.

The occurrence of prodromal symptoms in the days immediately preceding the major illness, in contrast to a definite first phase, was encountered in 27 per cent of 250 paralytic cases, but in only 9 per cent of 100 nonparalytic ones The frequency, symptomatology, and duration was more striking in adults than in children Listlessness, anorexia, and irritability were common in all age groups, but headache, pain, hyperesthesia and paresthesia were more frequent features of the symptomatology in those over 15 years

The differences in symptomatology and the greater difficulty in establishing a diagnosis in adults who often present an insidious onset with a bizarre picture early in the course is brought out by data on the day of hospitalization of para-

lytic cases Thus, of 250 paralytic cases, 81 per cent of those aged 2 to 4 were hospitalized during the first four days of the major illness, and 19 per cent on the fifth day or later. In contrast, 57 per cent of those over 20 were hospitalized during the first four days, and 43 per cent later than this time

To these two patterns, "childhood" and "adult," a third type [illegible] be added, the "infantile" one, encountered in those under [illegible] particularly under six months. These cases, the subject of [illegible] England[22] and New York City[23] frequently show relatively [illegible] characteristic meningeal signs are often absent, in spite of [illegible] the children have severe paralytic involvement The diffi[illegible] a diagnosis of nonparalytic poliomyelitis in this age groups [illegible]

Finally, as to the incidence of the different clinical types [illegible] nonparalytic, paralytic, and bulbar statistics are difficult [illegible] of the variable criteria used in defining a nonparalytic case [illegible] variable practices in different communities as to the report[illegible] cases at all. Nevertheless, with these defections in mind I [illegible] sent, first, data from New York City* and from Connecticut [illegible] illustrate certain trends in different age groups

FIGURE 3 shows the data for 2053 cases occurring in New Y[illegible] the period 1951 to 1953 Although attack rates in th[illegible] regularly low as compared to the 1-to-4 and 5-to-9 year o[illegible] cent of paralysis occurred in this youngest group of 25 [illegible] diagnosed as spinal paralytic, 12 per cent as bulbar [illegible] Greenberg[24] point out, the low incidence of nonparalytic cases is probably related to difficulties in diagnosis in the young infant Paralytic cases in New York City were next highest in the 1-to-4 year olds 71 per cent the lowest incidence of paralysis occurred in the 10-to-14-year age group 35 per cent The percentage then rose steadily in the older age groups to 75 per cent in those 30 and over Nonparalytic cases, on the other hand, were highest in the 10 to 14-year olds and lowest in infants and in the oldest age groups Bulbar cases were low in the young children, but more or less the same for those over 5, increasing slightly in the over-30 age group

The figures for Connecticut (FIGURE 4) show similar trends The nonparalytic cases were low in those under 5, rose to 60 per cent at 5 to 9 and main[illegible] ler age groups, in which [illegible] more prominent [illegible] 5) which, noted above, had a predominantly infantile age distribution similar to that in New York City in 1916, and quite unlike that for New York and Connecticut in the last few years, nevertheless show surprisingly similar trends in the different age groups[19]

The data for the three areas given in FIGURES 3, 4, and 5, in all of which the highest percentage of paralytic cases occurs in children under five and in adults, would seem to be in conflict with that based on "virgin-soil" epidemics pre-

* I am indebted to Doctor Morris Greenberg Director, Bureau of Preventable Diseases New York City Department of Health and to Doctor James C Hart Director Bureau of Preventable Diseases Connecticut State Department of Health Hartford, Conn , for making these data available to me

NEW YORK CITY 1951-'53
2053 CASES

TOTAL PARALYTIC
SPINAL
NONPARALYTIC
BULBAR & BULBOSPINAL
PERCENT
AGE <1 1-4 5-9 10-14 15-19 20-29 30+

FIGURE 3 Clinical type of poliomyelitis related to age per cent nonparalytic, spinal paralytic, and bulbar cases in each age group, New York City, 1951 to 1953 (Data from Doctor Morris Greenberg, Director, Bureau of Preventable Diseases, New York City Department of Health)

CONNECTICUT 1951-'53
1064 CASES

TOTAL PARALYTIC
NONPARALYTIC
SPINAL
BULBAR & BULBOSPINAL
PERCENT
AGE <1 1-4 5-9 10-14 15-19 20-29 30+

FIGURE 4 Clinical type of poliomyelitis related to age per cent nonparalytic, spinal paralytic, and bulbar cases in each age group, Connecticut 1951 to 1953 (Data from Doctor James C Hart, Director, Bureau of Preventable Diseases, Connecticut State Department of Health, Hartford, Conn)

sented earlier, which indicated that young children are relatively resistant to paralysis, compared to older age groups No completely satisfactory explanation for this paradox is at hand A possibility exists, however, that in populations such as those in New York City and in Connecticut, which are made up [illegible]

"heterologous" antibodies As a result of such immunity [illegible] suffers a modified, nonparalytic attack rather than a paralytic one [illegible] currence of heterologous immunity in man has been postulated [illegible] and recent laboratory data supply evidence that the three types [illegible] virus share common antigens Heterologous neutralizing antibodies have been [illegible] to develop following infection in humans[25] and, recently [illegible] tests have demonstrated relationships between the three types [illegible] known that the incidence of neutralizing antibodies to more than [illegible] creases with age In addition, there is experimental evidence [illegible] of cross-immunity between types of poliomyelitis viruses [illegible] session of heterologous antibodies might modify the clinical [illegible] tion is suggested by recent work reported by Sabin In [illegible]

[illegible] either [illegible]

[illegible]

[illegible] and his [illegible] workers

found that there was considerably *less paralysis* on oral challenge with the virulent type 1 Mahoney strain, than occurred in control animals which had never

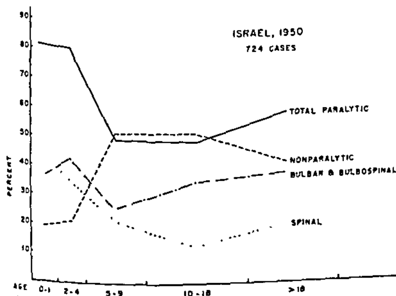


FIGURE 3 Clinical [illegible] percent nonparalytic, spinal paralytic, and bulbar

been exposed to type 2 Y-Sk virus. It is significant, as pointed out by Sabin that *none of the animals with Y-Sk antibodies was protected against infection* with type 1 virus, for all developed antibodies, but there was some protection against paralysis—*i.e.* the infection was modified and was milder. It seems *not unlikely* that, in the human population, the vulnerability of the young child, as far as paralysis is concerned, might be due to the larger number for whom the clinical attack, as a result of a virulent epidemic strain, represents the first infection [illegible]
nonparalyt [illegible]
possession [illegible]

It is recognized that a multitude of largely unknown factors complicate the picture and no simple answer is adequate. In fact, as with other problems associated with the effect of age on the host response to infection with poliomyelitis virus, the questions one raises are largely unanswerable, but nevertheless provocative.

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IMMUNIZING INFECTIONS WITH POLIOMYELITIS VIRUSES AND SEROIMMUNE PATTERNS IN SOUTHERN LOUISIANA*

By John P. Fox, Henry M. Gelfand, Pravin N. Bhatt, Dorothy R LeBlanc, and Donald P. Conwell

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The relative infrequency of overt disease, as compared with infection, has long imposed a serious barrier to full understanding of the mechanisms involved in, and the factors which influence, the dissemination of poliomyelitis viruses As less cumbersome laboratory methods have been developed, increasing efforts have been made to chart the occurrence of past infections in selected population samples by means of serologic surveys[1, 2, 3] and, in a few cases, by survey of serially collected sera, to discover the frequency of immunizing infections within defined periods of time.[4, 5] Efforts have also been made to discover persons actually harboring virus Poor environmental sanitation, low economic status, and large families have been named as factors contributing to early development of immunity.[3] Also, much evidence has accumulated to show that the probability of harboring virus increases with proximity to known, overt cases, and is maximum in child household associates of such cases [6, 7]

The work described in this paper relates to populations in southeastern Louisiana and was divided into two parts The first concerned itself with determining the extent of infection within the households of patients with clinically diagnosed disease occurring in late 1951 and in 1952. This project has been completed and is being reported in full detail elsewhere[8] The objective of the second part was to determine when, and under what circumstances, the average individual, selected without reference to overt disease, acquires his immunizing infections A corollary development, the delineation of patterns of seroimmunity within the study group, may reflect the patterns in the communities from which the group was selected This second project is being reported now only in preliminary fashion

Infections within Households of Poliomyelitis Patients

During the period from September 1951 through November 1952, specimens were collected from all members of 31 households that contained, in each instance, one or more patients admitted to the Charity Hospital Poliomyelitis Center in New Orleans within the first week of an illness diagnosed clinically as poliomyelitis This center serves the southern third of Louisiana, and nearly half of the households were outside the city of New Orleans. The specimens included single stools and sera taken as soon as possible after admission of the patient to the hospital and second sera collected about six weeks later Attempts were made to isolate and identify cytopathogenic viruses from all stool specimens and to demonstrate quantitatively the presence of neutralizing antibody to all three types of poliomyelitis viruses in the paired sera Nearly all

* This work was aided by a grant from the National Foundation for Infantile Paralysis, Inc, New York, N Y

of this work was done using tissue cultures of the plasma-clot type conta[illegible] skin muscle tissues obtained from premature infants dying soon after [illegible] The specific techniques, patterned closely on those developed by Enders [illegible] Two of the [illegible] virus and an [illegible] households [illegible] myelitis infection [illegible] ther discussion, therefore, will be restricted to the remaining 26 house[illegible] [illegible] virus Type-specific diagnosis of the remaining eight patients [illegible] by diagnostic or compatible serology and by the finding of virus [illegible] [illegible] 125 [illegible] the [illegible] stool specimen collected.

Since, for obvious reasons, virus may not have been recovered from all infected associates, serologic evidence of infection also was sought An analysis of the results of titrating the paired sera from the entire group for neutralizing antibody to the household virus type is presented in TABLE 1 The titrations involved testing serial fourfold dilutions of serum against approximately 100 tissue-culture infectious doses of virus and inoculating 2 cultures per serum dilution Differences in titers of simultaneously tested sera greater than four-

TABLE 1

DISTRIBUTION OF MEMBERS OF HOUSEHOLDS INFECTED WITH TYPES 1 OR 2 POLIOMYELITIS VIRUS BY CLINICAL STATUS, PRESENCE OF VIRUS IN STOOL AGE AND CHANGE IN TITER OF HOMOLOGOUS NEUTRALIZING ANTIBODY REVEALED BY TITRATION OF PAIRED SPECIMENS SERUM

Category of persons	Age group (yrs.)	Total number of persons	Number of pairs of sera tested	Number of persons whose paired sera showed homologous antibody titers as indicated				
				Rise > 4X	Fall > 4X	No significant change with		No antibody*
						Titers of 1:120 or >	Titers of < 1:120	
Patients	All	32	28	10		17	1	
Contacts shedding virus	All	45	32	9	1†	19	3	
Contacts without demonstrated virus in stools	0–4	5	5	4				1
	5–9	12	12	3	1†	3	4	1
	10–19	10	10	1	1†	4	3	1
	20 plus	51	45	6		18	21	3
	All	78	75	14	2	25	24	6

* No antibody in 1:10 final dilution of either serum specimen on screening test nor in 1:4 dilution when retested

† Individuals from household No. 18 the second serum specimens from whom were collected after an interval of six months

TABLE 2

DISTRIBUTION OF FAMILIAL ASSOCIATES OF PATIENTS IN 26 HOUSEHOLDS INFECTED WITH TYPES 1 OR 2 POLIOMYELITIS VIRUS BY AGE AND EVIDENCE OF CURRENT INFECTION

Age group (yrs)	Total number of associates	Infection status of household associates							
		Definite*		Probable**		Possible†		Probably not‡	
		Number	Per cent§	Number	Per cent§	Number	Per cent	Number	Per cent§
0–4	32	27	84	31	97	31	97	1	3
5–9	24	12	50	16	67	19	79	5	21
10–19	14	4	28	6	43	10	71	4	28
20 plus	53	2	4	8	15	26	49	27	51
All	123	45	37	61	49	86	70	37	30

* Persons from whose feces virus was isolated

** Includes persons from whose feces virus was isolated plus those manifesting a diagnostic rise in titer of homologous neutralizing antibody

† Includes persons from whose feces virus was isolated plus those manifesting a diagnostic rise in titer of homologus neutralizing antibody plus those manifesting titers high enough to be considered compatible with recent infection

‡ Includes those who remained nonimmune during the period of observation plus those whose titers were low enough to suggest immunity resulting from a past infection

§ Per cent was computed on the basis of the total number of associates even though not all furnished both stool specimens and paired serum specimens

fold have been regarded as of probable diagnostic significance It will be noted that, of 60 certainly infected individuals (patients or contacts shown to be shedding virus), whose paired sera were tested, 20, or 33 per cent, manifested such a change in titer Of the remainder, 90 per cent, or all but four, manifested serum titers of 1:320 or greater, or high enough to be considered compatible with current infection In contrast, the remaining contacts, from whose stools no virus was obtained, showed significant changes in titer in only 16 instances, or 21 per cent, and "high" titers in only 25 instances, or 33 per cent Further, the changes were relatively more frequent in persons under 10 years of age

In TABLE 2, all of the household associates have been distributed by age and in accordance with the available evidence as to their infection status If one cumulates into one group the associates found to be shedding virus with those showing a probably diagnostic change in antibody titer, the over-all proportion of almost certainly infected associates rises to 49 per cent Forty-seven of the 61 such associates were under 10 years of age The remaining associates have been classed as only possibly infected on the basis of high antibody titers (21 per cent) or almost certainly not infected (30 per cent) These 2 groups together included 45 persons 20 years or older, but only 9 below age 10 Included in the "not infected" group are 6 persons (3 children and 3 adults) whose sera, as shown in TABLE 1, contained no antibody to the household virus type and who must, therefore, be regarded as nonimmunes who escaped infection during the household episodes As such, they constitute exceptions to the general conclusion indicated by the over-all results, *i e* that, when an overt case of poliomyelitis occurs in a household, the other susceptible members are usually

infected concurrently. The probability of their being infected is of course inversely related to age and approaches 100 per cent in the zero to four year old group

Infections in Households with Newborn Infants

While evidence such as that just presented suggests that [illegible] likely to occur in persons associated with patients with overt [illegible] bear on the problem of when, and under what circumst[illegible] immunizing infections occur To approach this problem [illegible] selected as the one certainly nonimmune segment of the [illegible] tinued close observation of which should yield inform[illegible] sired More than 150 infants were recruited originally [illegible] the period of April through November of 1953 They [illegible] equally as possible with respect to race or socioeconomic [illegible] lower, and white upper), to birth order (first, second or [illegible] higher), and to area (New Orleans and Baton Rouge as urban [illegible] high past incidence of poliomyelitis, respectively, and [illegible] semirural character and low past incidence of disease)

As each infant was admitted to the study, a basic [illegible] containing all possible information of future epidemiologic [illegible] line sera were taken from all members of the household [illegible] after, the household was revisited to obtain an interval [illegible] logic history and stool and blood (heel-puncture specimens [illegible] child These specimens have been processed, using [illegible] ods,[10] on as current a basis as possible Evidence of infection, so far as [illegible] first indicated by seroconversion, has been the signal for immediate return to the household for additional information and for pertinent specimens from [illegible] of 1954 the first [illegible] evidence of [illegible] It is planned [illegible] study routine volun[illegible] nts

This study has been in progress for nearly 2 years, during which time only 11 households have been lost, 4 because of noncooperation and 7 because they moved out of the area The present study group embraces 146 households containing 150 original index children (4 sets of twins), 21 recently born younger siblings who are being followed in the same manner as the index children, and 670 older household associates The average period of observation per household and original index child is 16½ months (range 9 to 21 months) During this time, a number of interesting observations have been made

A total of 65 immunizing infections have taken place among 150 index children While 4 children have had multiple infections (3 with 2 virus types and 1, at age 17 months, having had his third infection), each infection or conversion is treated in the following as a separate event Further, seven of the

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All	123	45	37	61	49	86	70	37	30

[illegible]

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TABLE 3

DISTRIBUTION OF 58 IMMUNIZING INFECTIONS IN INDEX INFANTS BY MONTH OF OCCURRENCE AND BY VIRUS TYPE

Month and year	Number of Infections with virus of type			Total infections
	1	2	3	
Nov '53			1	1
Dec '53			1	1
Mar '54			1	1
May '54			2	2
June '54		2	2	4
July '54		11	8	19
Aug '54	3	4	4	11
Sept '54	3		2	5
Oct '54		2	6	8
Nov '54	1	1	3	5
Dec '54*			1	1

* Observations for December are incomplete

infections are indicated so far only by the continued persistence of antibody long beyond that expected for antibody of maternal origin While such infections certainly took place during the first six months of life, the pertinent stool specimens either do not exist (in four instances the infants entered the study some time after birth) or have not yet yielded virus isolations, and it has not been possible to fix the precise time and age of infection The remaining 58 infections represent instances in which the new appearance of antibody has been observed Probably because our technique for virus isolation has been relatively insensitive,* the fact of seroconversion usually has been our first and most reliable indication of infection While virus isolates compatible in type have been made from at least one stool specimen (usually that collected with the first positive blood specimen), in 37 of the 48 instances in which the stools have been examined, the full story as to time and duration of fecal infection will not be known until the pertinent specimens have been re-examined in monkey-kidney cell tubes[11] Except as indicated, further reference will be made only to these 58 "temporally defined" infections

Initial interest is in the time that these infections occurred in terms of both calendar time and age In TABLE 3, the infections are distributed by calendar month and by virus type While some of the seven undefined infections (not included in the table) may have taken place as early as the summer of 1953, all of these and three of the defined infections certainly occurred before April of 1954 A new season was perhaps initiated in May of 1954, when two conversions were recognized Since then, there have been four or more conversions in every month but December, for which the returns are incomplete July and August represented the months of maximum frequency. In TABLE 4, distribution is by age of the converting infants While the preponderance of infections took place in the age span of 10 to 16 months, the great bulk of the infants were

* In order to circumvent the high degree of toxicity of stool extracts for HeLa cells it has been necessary to permit only a brief period of contact between the cells and the stool inoculum and to follow that with a thorough washing of the cultures

TABLE 4

DISTRIBUTION OF 58 IMMUNIZING INFECTIONS IN INDEX INFANTS BY AGE AT WHICH INFECTION OCCURRED

Age in months	Number of infections	Age in months	Number of infections
4	2	14	[illegible]
5	1	15	[illegible]
6	1	16	[illegible]
9	2	17	[illegible]
10	4	18	[illegible]
11	8	21	[illegible]
12	9	22	[illegible]
13	3	23	[illegible]

born into the study during the 4-month period, June through Se[illegible] and were necessarily of closely similar ages during the sum[illegible] infections were most frequent. While this bunching of [illegible] seasonal and age basis is probably largely determined by [illegible] virus dissemination, an age-related increase in risk of exposur[illegible] bility Observation of the newborn younger siblings who [illegible] ing the study, because of the resulting increased diversific[illegible] relation to season in this early period of life, should provide more [illegible] this point. Of considerable interest is the fact that 11 infections [illegible] 7 undefined, temporally) took place in the first 6 months of life [illegible] the undefined instances, it can be stated definitely that maternally derived antibody was still present at the time of infection

As shown in TABLE 3, type 3 infections were most common (31 infants) type 2 [illegible]

[illegible] the 10 clinical cases coming from the study area and typed in 1954 Five of these were with type 3, 3 with type 2, and 2 with type 1 virus

The occurrence of infections with reference to area and racial or socioeconomic group is shown in TABLE 5. The areas differ chiefly with reference to virus types encountered More than half of the type 2 infections occurred in the Evangeline area, whereas six of the seven type 1 infections were in New Orleans Only type 3 infections were equally distributed When race and economic status are considered, possibly significant differences also are encountered Conversion rates among the 68 Negro and 40 white index children from the lower economic group were 37 and 32 per cent, respectively, while that for the 42 children from the white upper group was a rather surprising 48 per cent A partial explanation of this may lie in the fact that all but 3 of the latter children had older siblings, whereas the Negro and white-lower index children included 18 and 12 first-born infants, respectively The considerable importance of family size as a factor influencing the occurrence of infection is indicated by the fact that, of 33 first-born index children in the entire study group, only 8, or 24 per cent, converted, whereas of 117 children with one or more older siblings 50, or 43 per cent, experienced immunizing infections

TABLE 5

DISTRIBUTION OF 58 IMMUNIZING INFECTIONS IN INDEX INFANTS BY AREA, RACIAL OR SOCIOECONOMIC GROUP, AND VIRUS TYPE

Race or socioeconomic status	Virus type	Number of conversions in area indicated			
		Evangeline	Baton Rouge	New Orleans	All
Negro	1	0	0	1	1
	2	4	1	0	5
	3	7	7	5	19
	All	11	8	6	25
White lower	1	0	1	2	3
	2	1	1	4	6
	3	0	1	3	4
	All	1	3	9	13
White upper	1	0	0	3	3
	2	6	3	0	9
	3	3	3	2	8
	All	9	6	5	20
All	1	0	1	6	7
	2	11	5	4	20
	3	10	11	10	31
	All	21	17	20	58

As previously mentioned, return visits were made promptly upon discover of the fact of infection in order to obtain additional specimens, both of bloo and of feces, from household and other contacts, and these visits also added in formation of both clinical and epidemiologic interest Most of the stool speci mens and a few of the sera remain to be examined. The serologic results so

served in households with clinical cases, purely immunizing infections also occur on a household basis

The pathways by which infection gains entrance into the household thus become of predominant interest Evidence just presented suggests that, in a general way, older siblings of infants help to bring infection into the household Investigation of specific instances of infection have yielded bits of additional information In four instances, conversions took place simultaneously in pairs of, or, in one case, in a group of three households among which frequent visiting occurred In one instance, the index infant, an only child of immune parents, ... to play in a back yard con-
erved
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ion of

an index child been related to instances of paralytic disease.

Evaluation of the clinical information obtained poses a problem, since the special interrogation at the time of revisiting, over and above that conducted during the regular monthly visit, constitutes an inescapable bias. Certain

statements, however, can be made. Temporally defined infections have occurred in 58 index infants and in 56 older associates, or a total of 114 members of our study group. Retrospective evidence has been obtained that, at an appropriate time, 23 of these persons experienced minor but rather nondescript febrile illness associated with one or more of the following signs and symptoms: listlessness, headache, vomiting, constipation, myalgia, and stiff neck. In one such instance, a 22-month-old index child refused to walk or use his arms for a period of 4 days. In no instance, however, has infection been associated with illness which, at the time of its occurrence, was considered to suggest infection with poliomyelitis virus.

Patterns of Seroimmunity

Within certain limits, the members of the households containing index infants constitute samples of the communities within which they reside. Specifically, while the households were selected to represent certain attributes of possible importance to the spread of poliomyelitis viruses, the representation achieved was certainly not proportional. Also, there is an obvious bias stemming from the selection only of households with newborn infants that is largely responsible for a paucity in the study of persons in the 10-to-19 and 30-years-and-over age brackets. Nevertheless, the seroimmunity patterns indicated in TABLE 6 are of some interest.

It should be noted that the pattern, as of admission to the study in 1953, is basically presented, but that changes in the pattern representing the status in April and May of 1954 are indicated by percentage figures in parentheses. Attention is called first to the patterns for type-specific antibody within the entire group. These patterns are nearly identical, and they share two important, common attributes: (1) there is a sharp upward swing in per cent of persons immune after age three from an average of about 30 per cent to 60 per cent or more, and (2) near peak percentages (84 to 86 per cent) are reached in the second decade. One may also note that, in the interval between collection of the admission and of the first "annual" sera, except for single persons in the 5-to-9- and 20-to-29-year age brackets, all infections occurred in children under 5 years of age, and most of these were with type 3 virus.

The remainder of the table attempts to present a composite picture of the immunity pattern to all three virus types with reference to racial or socioeconomic group and to area. A footnote to TABLE 6 explains the derivation of the figures. Chief interest lies in the clearly delayed build up of immunity in the white upper economic group reflected in the low figure for over all immunity (62 per cent), as compared with those for the Negro and white lower groups (77 to 79 and 74 per cent, respectively). The patterns for New Orleans and for the Evangeline area are very closely similar with over all figures of 71 to 73 and 75 to 76 per cent, respectively. Considering that it represents an area of significantly higher past incidence of disease, the pattern for Baton Rouge differs to a *surprisingly small degree but in the expected direction of lesser over all* immunity (69 and 70 per cent) and of slower build up of immunity with age which is most evident in the 10-to-29 year span. Information as to the proportionate distributions of the populations of these areas with respect to race

TABLE 6

PER CENT OF HOUSEHOLD ASSOCIATES OF INDEX INFANTS SEROIMMUNE TO POLIOMYELITIS, BY RACIAL OR SOCIOECONOMIC GROUP, BY AREA AND BY AGE ON ADMISSION TO STUDY IN 1953

Age group (yrs.)	Number of persons							Per cent of 1953 and of (1954) sera positive†								
	From group indicated*			From area indicated**				From all areas and groups for antibody type			For all antibody types‡ from					
											Group indicated*			Area indicated**		
	Negro	Wh.-lo.	Wh.-up.	N.O.	B.R.	Ev.	Total	1	2	3	Negro	Wh.-lo.	Wh.-up.	N.O.	B.R.	Ev.
1	14	7	12	16	9	8	33	30	18	18	26	22	14	27	15	21
2	25	9	9	18	13	12	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
3	21	15	14	20	15	15	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
4	17	7	7	11	11	9	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]	[illegible]
5–9	44	34	35	40	36	37	[illegible]	[illegible]	[illegible]	[illegible] [illegible]	[illegible] [illegible]	[illegible]	[illegible]	[illegible]	[illegible] [illegible]	[illegible]
10–19	39	24	7	33	21	16	70	86	84	86	91	89	52	93	78	79
20–29	69	50	38	58	47	52	157	87	90	87 (88)	96 (97)	89	75	88 (89)	80	95
30+	74	36	63	73	46	54	173	86	93	90	93	92	84	86	90	93
All	303	182	185	269	198	203	670	73	72	71 (73)	77 (79)	74	62	71 (73)	69 (70)	75 (76)

* Wh.-lo. = white lower, Wh.-up. = white upper
** N.O. = New Orleans, B.R. = Baton Rouge, Ev. = Evangeline

and economic status would be of obvious interest at this point, but is not at the moment available.

Comment

The observations of households with overt cases of disease chiefly extend a considerable number of similar previous observations[6] and help to emphasize the fact that, when the disease poliomyelitis strikes an individual, nearly all of his then susceptible household associates are concurrently infected. Since, unfortunately, all sera collected in this study were inactivated immediately upon separation, no search could be made for circulating virus. By making such tests and relating positive findings to the age of the index case, Bodian and Paffenbarger[7] have obtained evidence that young members of a household are infected, as a rule, before older members.

It should be emphasized that the observation of newborn infants and their household associates is still in an early stage and that even the results reported for the average 16-month period of observation completed are of preliminary nature only, since much of the material collected remains to be examined for

the first time. Many indicated re-examinations have not yet been made. The problem of determining the time and circumstances under which the usual immunizing infection occurs is vital to the basic understanding of the dissemination of poliomyelitis viruses. The present study constitutes the obvious but necessary approach to the problem. Unfortunately, however, it is nearly unique. The only similar study so far reported was conducted on a very small [illegible]

[illegible]

sociates, (4) of 114 infections in index children and associates documented as to time of occurrence, only 23 yielded retrospective histories of suggestive minor illness and none experienced disease clinically recognized at the time as poliomyelitis; (5) in only 2 of 55 households could infection be linked, even possibly, to the occurrence of paralytic disease, yet (6) the distribution of infections in the study group by virus type paralleled closely that of patients admitted to the hospitals from the study areas in the same period.

Completion of the examination of material already collected and continued observation of the study group should provide even more interesting information. While evidence already obtained indicates that maternally derived antibody rarely persists to the sixth month, titration of the mass of heel blood specimens collected serially from birth should define precisely the rate and time of disappearance of such antibody. Utilization of more sensitive techniques for isolation of virus should permit more accurate estimations of the period during which virus is shed in the feces. Coupled with quantitative antibody determinations, such methods should also provide evidence as to the frequency of reinfection of seroimmune individuals under conditions of known household exposure. As increasing numbers of infections are detected, statistically firmer bases should be developed for evaluating the influence of such factors as race or socioeconomic status, family size, and regional or area environment.

Consideration of the study group as a valid population sample is open to some question. Nevertheless, within the study group, an inverse correlation was shown between economic status and rate of development and over-all extent of seroimmunity. Considering the study group as a whole, the percentage of immunes rose sharply after age three and reached near maximal figures in the second decade. This pattern, by currently accepted theory, fits well with the relatively low past incidence of disease in the bulk of the composite study area.

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Discussion of the Paper

DOCTOR JOHN R PAUL (*Section of Preventive Medicine, Yale University School of Medicine, New Haven, Conn*) Doctor Fox reports the kind of a survey that I wish I had done myself He has given us the opportunity to see when infants living under the conditions of his experiment acquire their first infection In this connection, the phenomenon of the infant becoming infected as it is losing, or has just lost, its maternal antibody would seem to me to be important If the child becomes infected at this time, is it a fortunate thing? Does this partial resistance on the part of the infant bear any relationship to the ratio of apparent to inapparent infections in the very young, or to the fact that the infection is milder in infants than in children who are older? Is that not what happens so frequently in a population such as that in Cairo, Egypt, where the reported incidence of poliomyelitis is very low and practically no cases are reported in native-born citizens who are over the age of six years? In contrast, in the northeastern parts of the United States, we witness the fact today that many children are being born without maternal antibodies as a result of the fact that a whole generation of susceptible mothers have reached the child-bearing age without having had the benefit of previous exposures and infection, whether apparent or inapparent The degree to which this is true can be appreciated when one realizes that more than 30 per cent of the cases of clinical poliomyelitis in New England occur in individuals over 15 years of age This gives us some idea as to how many children are being born there each year without maternal antibodies.

I congratulate Doctor Fox on this fine study

A LONGITUDINAL STUDY OF INFECTION WITH POLIOMYELITIS VIRUSES IN AMERICAN FAMILIES ON A PHILIPPINE MILITARY BASE, DURING AN INTEREPIDEMIC PERIOD*

By W. McD. Hammon, Ernest H. Ludwig, Gladys E. Sather, and William D. Schrack, Jr.†

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Epidemics of poliomyelitis with exceptionally high morbidity rates have from time to time involved United States and British military personnel and dependents on foreign bases. Natives in the same general area were frequently noted not to have an increased incidence. Americans have experienced a series of such incidents in the Philippine Islands, culminating in an unusually severe outbreak with about 50 cases on a United States Air Force Base in 1952. This outbreak, like many others, occurred at the same season of the year when epidemics occur in the United States. The Air Force requested advice regarding possible prevention of such episodes. A study to determine certain basic epidemiological facts was recommended, then undertaken.

By conducting a longitudinal study, it was hoped that one fact in particular might be definitely determined, *i.e.*, whether a person possessing antibody to any one type of virus might be reinfected and be a virus carrier. If this did not occur, association with natives, excepting possibly very young children, could be ruled out as one of the increased hazards.

This report is preliminary and covers results available to date on only one aspect of the project—the incidence and pattern of infection found during a four-months' period, August through November 1953, in two samples of dependent families.

Each sample contained approximately 100 families. These families included, in many instances, a female Filipino domestic servant. The two samples were selected to represent "old" and "new" residents. During the previous epidemic, it had been noted that a disproportionately high incidence of cases had occurred among the relatively newly arrived, so a higher level of immunity was generally assumed for the older residents. All in Group 1 had been resident on this post during the 1952 epidemic, many for a longer period of time. All in Group 2 had arrived since the termination of that outbreak, and many were very recent arrivals.

Methods

Practically every family selected in each sample volunteered to participate, when approached, and nearly all remained faithful throughout. Visits to the project's special clinic building were made every two weeks, as shown in TABLE

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc., New York, N. Y. These [illegible]

63: 527-550

12 GEAR, J 1952 Immunity to poliomyelitis Ann Internal Med 37: 1-22.

Discussion of the Paper

DOCTOR JOHN R. PAUL (*Section of Preventive Medicine, Yale University School of Medicine, New Haven, Conn.*): Doctor Fox reports the kind of a survey that I wish I had done myself He has given us the opportunity to see when infants living under the conditions of his experiment acquire their first infection In this connection, the phenomenon of the infant becoming infected as it is losing, or has just lost, its maternal antibody would seem to me to be important If the child becomes infected at this time, is it a fortunate thing? Does this partial resistance on the part of the infant bear any relationship to the ratio of apparent to inapparent infections in the very young, or to the fact that the infection is milder in infants than in children who are older? Is that not what happens so frequently in a population such as that in Cairo, Egypt, where the reported incidence of poliomyelitis is very low and practically no cases are reported in native-born citizens who are over the age of six years? In contrast, in the northeastern parts of the United States, we witness the fact today that many children are being born without maternal antibodies as a result of the fact that a whole generation of susceptible mothers have reached the child-bearing age without having had the benefit of previous exposures and infection, whether apparent or inapparent The degree to ... 30 per ... luals ... being born there each year without maternal antibodies

I congratulate Doctor Fox on this fine study.

A LONGITUDINAL STUDY OF INFECTION WITH POLIOMYELITIS VIRUSES IN AMERICAN FAMILIES ON A PHILIPPINE MILITARY BASE, DURING AN INTEREPIDEMIC PERIOD*

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Epidemics of poliomyelitis with exceptionally high morbidity rates have from time to time involved United States and British military personnel and dependents on foreign bases Natives in the same general area were frequently noted not to have an increased incidence Americans have experienced a series of such incidents in the Philippine Islands, culminating in an unusually severe outbreak with about 50 cases on a United States Air Force Base in 1952 This outbreak, like many others, occurred at the same season of the year when epidemics occur in the United States The Air Force requested advice regarding possible prevention of such episodes A study to determine certain basic epidemiological facts was recommended, then undertaken

By conducting a longitudinal study, it was hoped than one fact in particular might be difinitely determined, *i e.*, whether a person possessing antibody to any one type of virus might be reinfected and be a virus carrier If this did not occur, association with natives, excepting possibly very young children, could be ruled out as one of the increased hazards

This report is preliminary and covers results available to date on only one aspect of the project—the incidence and pattern of infection found during a four-months' period, August through November 1953, in two samples of dependent families

Each sample contained approximately 100 families These families included, in many instances, a female Filipino domestic servant The two samples were selected to represent "old" and "new" residents During the previous epidemic, it had been noted that a disproportionately high incidence of cases had occurred among the relatively newly arrived, so a higher level of immunity was generally assumed for the older residents All in Group 1 had been resident on this post during the 1952 epidemic, many for a longer period of time All in Group 2 had arrived since the termination of that outbreak, and many were very recent arrivals

Methods

Practically every family selected in each sample volunteered to participate, when approached, and nearly all remained faithful throughout Visits to the project's special clinic building were made every two weeks, as shown in TABLE

TABLE 1
CLINIC APPOINTMENT SCHEDULE AND SPECIMEN COLLECTIONS

Specimen	Fortnightly visits								
	1	2	3	4	5	6	7	8	9
Blood	+		+*		+		+*		+
Throat swab	+	+	+	+	+	+	+	+	+
Rectal swab	+	+	+	+	+	+	+	+	+

* Omitted for children less than 3 years of age

1, and blood, throat swabs, and rectal swabs were obtained at the intervals indicated. Children under three were bled only three times, *i.e.*, on the first, middle, and last visit. In order to induce parents to permit regular participation by children, all children in the study groups 10 years of age and under were given gamma globulin in a dosage of 0.14 cc. per pound just after the first visit and, thereafter, at four-week intervals—a total of four injections Previous studies, already reported,[1] had shown that this amount of antibody did not prevent infection itself at a subclinical level, and previous and current studies[2] have shown that the antibody levels produced, though occasionally detectable at a 1:4 serum dilution three to four weeks later, do not confuse the serological [illegible]

Tests for virus were made in trypsinized monkey-kidney tissue cultures, and *agents isolated were identified as poliomyelitis if neutralized by one of three* specific monkey immune typing sera Neutralization tests were performed using similar tissue culture tubes, employing the serum dilution method, with 100 TCD_{50} of each type of virus Results in the tables are presented as the reciprocal of the serum dilution calculated to protect 50 per cent of the inoculated tubes

Complement fixation was performed by a modification of the plate method of Fulton and Dumball,[3] using antigen prepared from tissue cultures, concentrated and purified by the Svedmyr, Enders, and Holloway technique[4] Two units of complement and two units of antigen were employed, and readings in the tables represent the reciprocal of the highest serum dilution giving a four-plus reaction This is a standardized test which we have performed on more than 1000 other sera from poliomyelitis studies prior to undertaking this project[1, 2]

Results

During the entire four months of study, only one reasonably certain case of mild paralytic poliomyelitis occurred on the entire base—an adult—not in the study group Not one person in the two samples discussed in this paper was even suspected on a clinical basis

Since testing is still incomplete, we can give no reliable infection rates at present Numerous infections, however, resulting from all three recognized

TABLE 2
FAMILY 2-55

Age Sex	Virus type	Dates of specimens										Date virus isol Type
		Comp fix					Neutralization					
		8 4	9 1	9 29	10 27	11 24	8 4	9 1	9 29	10 27	11 24	
40	1	0	0	0	0	0	32	—	—	—	32	
M	2	0	4	0	4	0	—	—	—	—	—	
	3	4	8	8	8	4	<4	<4	<4	<4	<4	
33	1	4	—	0	—	0	16	—	—	—	16	
F	2	4	—	4	—	4	32	—	—	—	32	
	3	4	—	4	—	4	16	—	16	16	8	
5	1	0	—	0	0	0	<4	—	—	—	2	
F	2	0	—	0	0	0	<4	—	—	—	2	
	3	0	—	0	4	4	<4	—	<4	64	256	
4	1	0	0	0	8	4	<4	—	—	—	—	11-10
F	2	0	0	0	16	8	4	—	—	—	—	3
	3	0	0	0	8	4	<4	<4	<4	256	512	
1	1	0		0		0	<4		—		<4	
F	2	0		0		0	<4		—		<4	
	3	0		0		8	<4		<4		512	
21	1	0	—	0	—	0	>4	—	—	—	—	
F	2	0	—	0	—	0	>4	—	—	—	—	
Maid	3	0	—	0	—	0	128	—	64	—	128	

virus types, with type 3 predominating, have been demonstrated by laboratory methods. Most tests have been of a serological nature, but several type 3 viruses have been isolated from rectal swabs. Since a very large number of other untypable agents was isolated, all isolation attempts were stopped over six months ago, until a method could be developed to identify and suppress these other agents which, we feared, might interfere with the main aims of the study.

Examples of what has been found in representative families will be presented in a series of tables, each containing the results of tests completed to date on all members of one family, including the native domestic, if she had been persuaded to participate.

In TABLE 2 is presented family 2-55, of Group 2 (newly arrived). Father and mother are presumed to be immune to type 1 virus because of their neutralizing antibodies (1:32 and 1:16, respectively) and the mother to types 2 and 3 also. They show no changes in titer for any virus for which tests were performed by either type test, so we assume they were not infected when the three children were. The low titer of type 3 C.F. antibodies shown by the father is unexplained but, as we shall point out again later, it is due, possibly, to another agent with a common antigen. The three children, ages five, four, and one, sho [illegible]

pino domestic, age 21, had neutralizing antibodies to all 3 virus types, as did all

TABLE 3
FAMILY 2-25

Age Sex	Virus type	Dates of specimens										Date virus isol Type
		Comp fix					Neutralization					
		8 3	8-31	9 28	10 26	11 23	8 3	8 31	9-28	10 26	11-23	
31	1	0	—	0	—	0	128	—	—	—	512	
M	2	0	—	0	—	0	<16	—	—	—	<16	
	3	0	—	0	—	4	<4	—	<4	64	256	
28	1	0	—	0	—	0	—	—	—	—	<8	
F	2	0	—	0	—	0	64	—	—	—	128	
	3	0	—	0	—	0	<4	—	<4	—	<4	
3	1	0	—	0	—	4	128	—	—	—	256	10 26
M	2	0	—	0	—	4	<4	—	—	—	<4	3
	3	0	—	0	—	4	<4	<4	<4	128	256	
1	1	0		0		0	<4		—		—	
M	2	0		0		0	2		—		—	
	3	0		0		8	<4		<4		128	

other domestics tested, and had no titer changes for type 3 virus by neutralization or C F tests In this family group, then, two apparently immune and one nonimmune adults were spared infection, though three children acquired infection at least one month before the study closed

In TABLE 3, family 2-25, also Group 2, is seen to have three type 3 infections involving a nonimmune father and two susceptible children Type 3 virus was isolated from the swab collected on October 26 from the 3-year-old Other specimens must be tested to obtain more accurate time sequence. The mother, though with no detectable type 3 neutralizing antibody, strange as it may seem, was spared She was near term when potentially exposed and gave birth in the hospital to a healthy baby on November 7th.

In TABLE 4 is presented family 2-111, also a newly arrived group. Two children had type 3 infections, both parents were without type 3 antibodies, but failed to become infected, and the domestic, an immune, showed no C.F. antibody responses

The next family selected, 2-180 (Group 2), TABLE 5, has mother, father, and both children originally without detectable antibodies to type 3 virus, then infected with type 3 virus Virus isolations confirm the serology on the two children (adults not tested) Only the Filipino girl, age 23, shows no evidence of infection, current or recent

TABLE 6 presents data on family 1-211 of Group 1 (older residents) consisting of father, mother, and a 22-months-old child We interpret these results to indicate infection of all with type 3 virus, at about the same time, although further tests on the November 4 specimens of blood and attempts to isolate virus may fix this more accurately The father appears to have had no previous antibody to types 2 and 3 (type 1 not yet tested) and shows no evidence of any recent infection, since he had no C F antibodies In fact, apparently he had not responded yet with C F antibodies to type 3 when bled last, but neutralizing antibodies had just begun to rise slightly His wife, however, ap-

TABLE 4
FAMILY 2 111

Age Sex	Virus type	Dates of specimens: Comp fix 8 5	9 2	9 30	10 28	11 25	Neutralization 8 5	9 2	9-30	10 28	11 25	Date virus isol Type
31	1	0	—	0	—	0	32	—	—	—	16	
M	2	0	—	0	—	0	128	—	—	—	64	
	3	0	—	0	—	0	<4	—	<4	—	<4	
29	1	0	—	0	—	0	64	—	—	—	64	
F	2	0	—	0	—	0	128	—	—	—	128	
	3	0	—	0	—	0	<4	—	—	—	<4	
5	1	0	—	0	0	0	<4	—	—	—	—	
F	2	0	—	4	4	0	<4	—	—	—	—	
	3	0	—	4	8	8	<4	—	<4	32	256	
3	1	0	—	0	0	0	<4	—	—	—	8	
F	2	0	—	0	0	0	<4	—	—	—	4	
	3	0	—	0	4	8	<4	—	4	—	1534	
20	1	0	—	0	—	0	>4	—	—	—	—	
F	2	0	—	0	—	0	>4	—	—	—	—	
Maid	3	0	—	0	—	0	>4	—	—	—	—	

TABLE 5
FAMILY 2 186

Age Sex	Virus type	Dates of specimens: Comp fix 8 10	9 7	10 5	11 2	11 30	Neutralization 8 10	9 7	10 5	11 2	11 30	Date virus isol Type
27	1	4	4	4	16	8	—	—	—	—	—	
M	2	4	4	4	16	32	—	—	—	—	—	
	3	0	0	0	8	8	<4	<4	<4	512	512	
26	1	0	0	0	8	8	—	—	—	—	—	
F	2	0	0	0	4	4	—	—	—	—	—	
	3	0	0	0	32	32	<4	<4	<4	>1024	1024	
4	1	0		0	0	0	<4	—	—	—	<4	10 5
F	2	0		0	4	0	<4	—	—	—	<4	3
	3	0		0	8	16	<4	<4	8	256	256	
20 mo	1	0		0		0	<4		—		4	11-2
M	2	0		0		0	<4		—		<4	3
	3	0		0		8	<4		4		1024	
23	1	0	—	0	—	0	>4	—	—	—	—	
F	2	0	—	0	—	0	>4	—	—	—	—	
Maid	3	0	—	0	—	0	>4	—	—	—	—	

parently had had a quite recent type 2 infection, with her neutralizing antibodies at a very high level and C F antibodies just beginning to fall She

an that of October 5 Between her last two specimens, however, her C F antibodies rose from 1 8 to 1:32, and her neutralizing antibodies appear to have

TABLE 6

FAMILY 1-211

Age Sex	Virus type	Dates of specimens										Date virus isol Type
		Comp fix					Neutralization					
		8 10	9 7	10 5	11-4	11-30	8-10	9 7	10 5	11-4	11 30	
27	1	0	—	0	—	0	—	—	—	—	—	
M	2	0	—	0	—	0	<4	—	<4	—	<4	
	3	0	—	0	—	0	<4	—	<4	—	16	
23	1	16	16	8	16	8	—	—	—	—	—	
F	2	64	32	32	32	16	>1024	—	>1024	—	>1024	
	3	16	16	8	8	32	64	—	32	—	128	
22 mo	1	0		0		0	<4		<4		<4	
M	2	0		4		8	<4		8		<4	
	3	8		8		32	<4		<4		512	

been rising, as evidenced by a 1:128 titer in the last one. These rises in both types of antibody we interpret as suggestive evidence in support of reinfection At the same time as these changes occurred in the sera of husband and wife, those of the child showed a neutralizing antibody rise from <1:4 to 1:512, and showed also a C F. antibody rise We have no explanation for his previous low titer of C.F. antibodies to type 3 except to suggest that it remained from a previous infection with an agent having a common antigen with type 3 virus, the existence of which we strongly suspect in this area from our experience with many other sera

Next, in TABLE 7, a few individuals are presented which represent interesting situations A 21-year-old mother (1-107), an older resident, exposed to a one-year-old with type 2 involvement, has evidence of previous type 2 infection with neutralizing antibody in her first specimen at a level of 1:16 She subsequently developed C.F antibodies to type 2, and shows a modest rise in neu-

TABLE 7

No	Age Sex	Virus type	Clinic visits										Expos virus type
			Comp fix					Neutralization					
			1	3	5	7	9	1	3	5	7	9	
1-107	21	1	0	—	0	—	0	—	—	—	—	>4	2
	F	2	0	—	4	—	16	16	—	128	—	64	
		3	0	—	0	—	0	<16	—	—	—	<16	
1-286	7	1	0	—	0	—	16	128	—	128	512	>1024	1
	F	2	0	—	0	—	4	<4	—	8	8	256	
		3	0	—	0	—	0	—	—	—	—	<8	
1-259	4	1	0	0	0	0	4	—	—	—	—	—	3
	M	2	0	0	0	0	8	—	—	—	—	—	
		3	0	4	4	64	16	<2	<2	<2	128	32	
2-64	3	1	0	64	32	32	32	<4	—	—	—	256	1
	F	2	8	64	64	32	32	>256	—	—	—	>256	
		3	0	4	4	4	0	<16	—	—	—	<16	

neutralizing antibody rose to 1:512, then to >1:1024 Although we interpret these and others of a similar nature to suggest reinfection strongly, virus isolation will furnish the most important proof, and tests for virus have not been made

A four-year-old (1-259) in the family with another type 3 infection shows a rather rapid rise and fall in neutralizing antibodies, an exception to the usual finding Next, a type 1 infection in a three-year-old (2-64) is interesting from the standpoint of infection occurring in a person previously immune to a recent type 2 infection, with residual C F antibodies (1 8) presumably from that infection The C.F response to the new infection gave essentially an equal response to both types of virus Time does not permit further presentation of the great variety of response patterns shown

In order to measure the influence of the 1952 epidemic and other previous experience in the Philippines on persons with longer residence, their serologic evidence of immunity, by age groups, has been compared with that of the newly arrived Group 2 Because of the special selection of the adult sera tested so far, they are omitted from consideration, but we have every reason to believe that sera from the children under 10 years of age represent a random sample from each group

In FIGURE 1, presented graphically by the front row of columns, are the percentages in each group found to have antibodies to each type of virus at the beginning of the study These are actually age-specific antibody rates per 100 The total number tested in each age group is shown by the figure on the top of the column It will be noted that a peculiar pattern is shown in Group 1 for

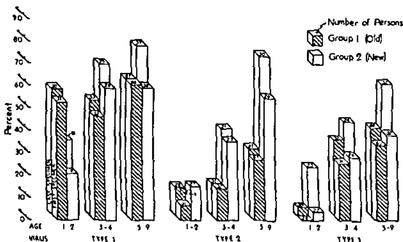


FIGURE 1 Percentage of children with antibody by type, age, and group

type 1 virus antibodies All ages have attained approximately equal rates of infection. From this and other even more convincing evidence, we conclude that type 1 virus was the epidemic strain of 1952 Many one-year-old children have type 1 antibody *and this type only, including one with* residual paralysis [illegible]

[illegible]

and the differences are in the same direction for type 3, although of lower magnitude.

When one looks for the changes in immune status *which occurred during* the four months of the study, as indicated by the difference in the heights of the columns in the front and back rows, it is noted that few in Group 1 became infected with type 1 virus, while more activity was observed in the newcomers, even in the 5-to-9-year age group, in which the percentage of immunes was equal *to that of Group 1 at the beginning* The same trend is seen for type 2 virus in the two older age groups of the newly arrived (Group 2), although proportionately *more* immune than those with *longer residence* (Group 1) For type 3 virus there is more evidence of infection occurring in both groups at all ages, confirming the family and individual observations shown earlier of more frequent type 3 infection. Again, however, the more immune group (newcomers) at each age showed the higher current infection rates It appears reasonable *to suggest, on the basis of these two observations on newcomers—the* higher clinical attack rates of 1952 and the higher laboratory demonstrated rates of inapparent infection of 1953—*that* newcomers are the more likely to become infected, but the reason does not appear to be that which has previously been offered, to the effect that newcomers are less immune. The new population actually contained a higher proportion of immunes by age than the older residents to two types of virus and even to type 1 virus in one age group

Complement fixation tests have been completed on essentially all persons in the two groups, so results on adults can be examined without fear of bias in selection Limited time prevents presentation of details, but results on children's sera tend to support those observed from neutralization tests, indicating that newly arrived persons were more heavily exposed to infection during their early months of living on the post

Adult figures by complement fixation show one thing not observed in available *neutralization tests, the latter data probably reflecting* total life experience Complement fixation for all three virus types shows that the wives in Group 1 (longer residence) had been more frequently infected in the relatively short period of time during wh[illegible] than had their husbands For example, only 17 per [illegible] 89 wives had elevated CF antibodies t[illegible] *me* nature for the other two types This large difference occurs despite the fact that practi*cally every* husband had resided on the base several months and frequently a year longer than had his wife This same difference in adult exposure is not

apparent among those only recently arrived. Evidence, therefore, of all serological tests fails to support the pre-existing epidemiological concept of the Philippines as a hotbed of poliomyelitis infection 12 months out of the year to all virus types, leading to higher infection rates than normally acquired at home and leading to an increased percentage of immunes. It points, instead, to modest infection and immunization rates affecting principally the newly arrived.

The longitudinal-prospective type of study reported here, yielding a long series of specimens on normal persons, has impressed us with several great advantages. After having previously performed similar serological and virus isolation tests on serial specimens from several hundred cases of poliomyelitis

tation of serological tests. It also permits one to select with some certainty those with and without infection prior to the current, recognized exposure, and has given suggestive evidence in support of reinfection. This finding we predicted a number of years ago on epidemiological evidence alone,[5] and the added evidence is quite welcome. To date, however, we have found no evidence of any Filipino sharing in a household infection or with serological evidence of an infection preceding that of other members in the family, and thus a probable contact source. Such negative evidence, in view of the fact that Filipino women usually serve as nursemaids for the American children and are in intimate contact, suggests that they may have a more solid degree of immunity than most of the American parents. This, in turn, we presume to be due to more frequent or repeated infections. In any case, we have no evidence yet to incriminate young adult Filipino domestics as carriers in a situation where they have been strongly suspect heretofore. Very few, however, have been tested for actual virus excretion as yet, and we may be assuming too much from the absence of serological changes.

One concept has been adequately confirmed by these studies—that poliomyelitis viruses of all three types may be prevalent and may produce numerous infections in a community without producing clinically recognized disease. In other words, in nature there are relatively nonpathogenic strains of virus carrying on an effective, harmless, immunizing process. A number of these strains have been isolated from our material and are available for study. It is interesting to speculate on whether such strains are more prevalent in countries where natives have fewer epidemics, less paralytic disease, and where a much higher proportion of their population is highly immune, through what we now may presume with more certainty to involve frequent reinfection. If passage through immunes has led to selection of such strains, we might then speculate further that "epidemic strains," such as the type 1 strain of 1952, might have been imported, perhaps from the United States, where many opportunities occur for passage of strains from susceptible to susceptible in series. Furthermore, friendships made among families on the ship *en route* to the Philippines might be assumed to be cultivated and nurtured intensely after arrival on the

base. The new segment of the population, together with the waiting husbands, continues its intimate contacts and behaves, for a while, quite differently from the rest of the population on the base. This involves group exploration and investigation of everything new and different in the strange environment. There is some support from observation for these latter assumptions on social patterns. Thus, a strain of virus imported by such a group, or acquired by one of its members soon after arrival, might demonstrate its activity excessively in this as-yet-unassimilated segment of the population.

A preliminary report of this kind on one small part of a study can hardly be expected to furnish all the answers, but certain patterns have been suggested of what we may anticipate will represent an exceedingly complicated and constantly changing biologic complex. Certainly, no retrospective epidemiologic study could hope to reveal the pattern, and we can expect only limited progress from a prospective study of but four months' duration and with only 30,000 specimens to be tested

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4 Sv [illegible] first on with Brun [illegible] L
5 H [illegible]

VIRUS EXCRETION AND ANTIBODY RESPONSE IN CLINICAL AND SUBCLINICAL CASES OF POLIOMYELITIS*

By Gordon C. Brown
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Previous to the advent of tissue culture techniques for the isolation and [illegible]

techniques Furthermore, serological studies, except for those involving the mouse adapted strains of type 2 virus, were almost precluded because of the tremendous numbers of animals involved [17-25] Since the tissue culture test tube has now replaced the monkey as a method for growing the virus,[26-32] almost unlimited horizons have been presented to the epidemiologist, with the result that, today, it is possible to acquire data of truly significant scope for the first time It is the purpose of this paper to report some results obtained in this manner

Clinical and subclinical cases, 1953 During 1953, a relatively small number of clinically diagnosed cases in Michigan were studied for the presence of virus in stationary tube cultures of Hela cells [33] Ten of 29 cases were classified as paralytic, and virus was recovered from seven of eight available fecal specimens (TABLE 1) In contrast, the agent was isolated from only 8 of 18 specimens obtained from nonparalytic cases Quantitative studies were made by direct titration of the stool specimens in tissue cultures where the titers ranged from 10^{-1} to 10^{-4} The average titer of virus from paralytic cases was found to be $10^{-2.8}$, while that from the nonparalytics was $10^{-2.7}$ The immunological type of each virus was determined by neutralization test technique with specific hyperimmune sera of monkeys and, except for one type 3, all viruses were found to belong to type 1.

Stool specimens from 134 familial associates of these diagnosed cases were tested for virus In families with index cases, confirmed as poliomyelitis by recovery of virus, 29 of 70 (41 per cent) associates were found to be subclinically infected In contrast, virus was recovered from only 4 of 64 (6 per cent) of the associates of cases who were negative for virus In all of these, the immunological type of the virus was identical with that of the index case The average titers of virus in stools were $10^{-2.9}$ and $10^{-2.1}$, respectively, for the two groups, with a range from 10^{-1} to 10^{-6} Two additional collections of specimens at weekly intervals demonstrated persistence of virus in essentially the same titer during this period of infectivity These results are of particular interest, since the individuals had received a doubled dose of gamma globulin prior to the study and show that neither the quantity nor the persistence of virus was affected by passively administered antibodies In an attempt to reveal the existence of chronic subclinical infections, however, 22 of the individuals known

* These studies were aided by a grant from the National Foundation for Infantile Paralysis, Inc , New York, N Y

TABLE 1

ISOLATION OF VIRUS FROM INDEX CASES

Diagnosis	Pos	Neg	No spec
Nonpar.—19	8/18	10	1
Par —10	7/8	1	2

ISOLATION OF VIRUS FROM FAMILIAL ASSOCIATES

Index case	Collection		
	1	2	3
Neg (11)	4/64	1/62	2/61
Pos (18)	29/70	27/71	27/70

to excrete virus previously were tested six months later, and all were found to be negative

Pharyngeal virus was isolated from 6 of 25 individuals with demonstrable fecal infection In all cases, the immunological types of virus in stool and pharynx were identical No virus was isolated from the throat swabs of 28 associates who had no poliomyelitis virus in their stools

The initial sera from 23 associates with subclinical infection were tested for viremia No viruses were isolated No special techniques however such as ultracent f

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cent) were found to have at least low-level antibodies to the homologous type of virus with which they were infected Only 8 of them (34 per cent) had antibody titers of 1 64 or better, and 11, or 48 per cent, had no detectable antibodies at all In contrast to these figures, only 5, or 12 per cent, of 32 uninfected associates were devoid of antibodies, while 27 (84 per cent) had titers of 1/4, and 24 (75 per cent) of the sera neutralized 100 tissue culture doses of virus in dilutions of 1/64 or higher These findings are in accord with previous observations, which have suggested that virus is less likely to become established in persons with type-specific antibodies [17] A marked rise in homologous antibody titer was observed in 65 per cent of the second blood specimens collected six weeks later from subclinically infected individuals It must be pointed out that

among the 11 persons showing a rise was 35-fold In contrast to these results, only 2 of 12 uninfected persons showed an increase in antibodies over the same period, and the average rise was only fourfold

Clinical cases, 1954 During 1954, 137 clinically diagnosed cases from Michigan were tested in HeLa cell cultures for the presence of virus In con-

TABLE 2

SEROLOGICAL STATUS AMONG FAMILIAL ASSOCIATES OF CASES OF POLIOMYELITIS

	Infected (23)			Uninfected (32)	
Antibody level of initial sera	1/4	12	(52%)	27	(84%)
	1/64	8	(34%)	24	(75%)
No antibodies		11	(48%)	5	(12%)
Increase in titer increment		15/23	(65%)	2/12	(16%)
		53		4	

TABLE 3

TISSUE CULTURE ISOLATION OF VIRUS FROM CLINICAL CASES OF POLIOMYELITIS IN MICHIGAN 1954

	Tested in				Total positive	
	HeLa cells		Kidney cell			
	pos	neg → pos		neg		
Par (60)	25	35	17	10	42	70%
N P (77)	11	66	11	42	22	28%
Tot (137)	36	101	28	52	64	46%

trast to the previous work, for which all tissue cultures were propagated in our laboratory, the culture tubes were obtained from the Tuskegee Institute in Alabama where, presumably, the same line of cells and the same techniques were used TABLE 3 shows that poliomyelitis virus was isolated from the stool specimens of 25 of 60 paralytic cases (41 per cent) and from 11 of 77 nonparalytics (14 per cent) Although many of these specimens were not collected as promptly, following onset, as in previous studies, the percentage of isolations was still conspicuously low Consequently, 52 of the specimens which were negative in HeLa cell cultures were tested in cultures of monkey kidney tissue * A total of 28 additional viruses have been isolated in this manner, 17 from paralytic cases and 11 from nonparalytics with a higher percentage of success from the former The total percentages of isolations thus became 70 from paralytics and 28 from nonparalytics, a much closer agreement with previous work Unfortunately, the conclusion must be reached that Tuskegee type HeLa cells, as used in these tests, are far less satisfactory than are monkey kidney cultures for the primary isolation of poliomyelitis virus from fecal specimens

The distribution of the immunological types of the 64 viruses is presented in TABLE 4 and shows an entirely different division than in the previous year The marked increase in the prevalence of type 3 virus is particularly significant Except for certain local areas during 1950, this virus has not been demonstrated [illegible]

appears to be due to this type of virus

* We are indebted to Doctor Herbert Wenner of the University of Kansas Lawrence Kans and to Doctor Frederick Robbins of Western Reserve University Cleveland Ohio for performing these tests

TABLE 4
IMMUNOLOGICAL TYPES OF POLIOMYELITIS VIRUS FROM CASES IN MICHIGAN, 1954

	1	2	3
Paralytic	23	3	16
Nonparalytic	10	5	7
Total	33	8	23

TABLE 5
SEROLOGICAL STATUS AMONG CASES OF POLIOMYELITIS, MICHIGAN, 1954

Initial sera	Virus isolated (44)			Virus not isolated (47)		
				1	2	3
Homologous antibody level	1/4	38	86%	25 (53%)	21	22
	1/64	19	43%	11 (23%)	7	8
No antibodies		6	13%	22 (46%)	26	25

The serological status of the cases is presented in TABLE 5. The initial specimen of blood was not always collected as promptly as in the 1953 study, and this considerable interval of time may explain the higher percentage of individuals having circulating antibodies at the time of the first bleeding. Thirty-eight of 44 infected persons (86 per cent) had an homologous antibody titer of at least 1/4, and half of these could be diluted to 1/64 and still neutralize the virus Only six infected persons had no demonstrable titer at the time of the first collection Many of these people had no antibodies to the other two types ... with the

in their initial sera Fewer of these sera, however, could be diluted as high as 1/64 and still neutralize the three types of virus ... one month after the ... iduals ... aralytic

cases developed antibodies than did the nonparalytics, and the increments in antibody titer were sixfold and fourfold for the two groups, respectively. This increment was considerably less than that found in the subclinically infected individuals, and can probably be explained by the fact that more of these persons already had antibodies by the time of the first bleeding This finding is similar to that of Miller and Wenner,[26] who described high neutralizing antibody titers in clinical cases as early as two days after the first symptom with very little subsequent change A smaller number of the persons from whom no virus could be isolated were shown to have developed antibodies during this period Most of these increases were in type 1 antibodies, and this serological evidence of infection should confirm the clinical diagnosis even though the agent

TABLE 6

INCREMENT IN ANTIBODY TITER AMONG CLINICAL CASES OF POLIOMYELITIS, MICHIGAN, 1954

	Virus isolated homologous	Virus not isolated		
		1	2	3
Par	22/30 (6 fold)	4/11 (5)	1/11 (8)	1/11 (3)
Nonpar	6/14 (4 fold)	6/36 (3)	3/36 (2)	1/36 (1)
Total	28/44 (6 fold)	10/47 (4)	4/47 (3)	2/47 (2)

TABLE 7

MIXED IMMUNOLOGICAL TYPES IN INFECTED FAMILIES, MICHIGAN, 1954

Case	Associate
1	1, 3, 3
1	3
1	3,3
1	3

itself could not be detected A serious question should be raised, however, con- [illegible]

and virus has been isolated from 70 Thirty-seven of these belonged to type 1, two were type 2, and 31 were type 3, showing a distribution almost identical with that of the diagnosed cases In general, the type of virus isolated from associates has agreed with that obtained from the case Four interesting exceptions, however, have been observed (TABLE 7)

In the first family, the virus from the case belonged to type 1, as did that of one of her brothers Two other siblings, however, were clearly type 3 subclinical poliomyelitis Three other families in which the cases were type 1 had associates with subclinical infections caused by type 3 virus The entire typing procedure has been repeated with identical results starting from the original stool specimens of all four cases and of all the associates in the first three families Serological studies have not been completed, but results to date suggest that some members of the families respond with antibodies to both types of virus These situations present a very interesting epidemiological oddity and prove that it is possible for at least two immunologically different viruses to infect individuals of the same family at the same time In addition, the fact that the clinical disease in all four families was caused by type 1 virus, while the subclinical infections were due to type 3, may have significance In spite of the observed increase in type 3 virus as a causative agent of the clinical disease, it appears to have less pathogenesis than does type 1 Type 2 virus has been considered for years in a similar position with its prevalence demonstrated by antibody surveys, yet rarely incriminated as causing the clinical disease

Summary

The results of virus isolations from clinical and subclinical cases of poliomyelitis occurring in Michigan during 1953 and 1954 are reported. The agent was isolated from a greater percentage of paralytic cases than from nonparalytics, but no significant difference in virus titer was observed. The distribution of immunological types of virus showed a marked increase in the prevalence of type 3 during 1954. The serological patterns for both paralytic and nonparalytic cases are presented, and they show that an increase in homologous antibodies occurs only about 65 per cent of the time. Many of the paralytic cases, however, had high antibody titers at the time of the first bleeding. Four families were found to have mixed infections with two different immunological types of virus. The significance of the findings is discussed.

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Discussion of the Paper

Doctor John O'H Tobin (*Department of Bacteriology, University of Minnesota, Minne*... similar to fi... percentage ... cent from nonparalytic In the la... *patients under 10 years than in the* ... litis epidemic was predominantly t... of this type In 1954, however,

*Tobin J O'H, A T Brunner P M Ellwood & J T Syverton 1954 Proc Cen Soc Clin Research 27 127 1954 J Lab Clin Med 44 941 94?

TABLE 1

SUMMARY OF THE RESULTS OF EMPLOYING CULTURES OF HUMAN EPITHELIAL CELLS (HELA) FOR THE ISOLATION AND IMMUNOLOGIC CLASSIFICATION OF POLIOMYELITIS VIRUS

Distribution of Immunologic Types Among First 810 Isolates

Specimen from	Total by year	Immunologic type		
		T1	T2	T3
1946	7	7	0	0
1948	59	59	0	0
1949	20	9	11	0
1950	54	21	1	32
1951	15	12	2	1
1952	85	77	0	8
1953	394	385	1	8
1954	176	52	43	81
Total	810	622	48	130
Percental number		77	7	16

distributed: of 182 isolations, 53 were type 1, 43, type 2, and 86, type 3 The percentage of isolations from paralytic and nonparalytic cases has so far been somewhat lower, 67 per cent and 30 per cent respectively. HeLa cells prepared in our laboratory and from Tuskegee were used for the 1954 isolations No difference was noted in the sensitivity of cells from either source to the poliomyelitis viruses

Antibody levels to the three virus types have so far been completed only for 1953 The range in antibody titer to type 1 virus was similar for paralytic and nonparalytic patients from whom type 1 virus was isolated In contrast to these findings, the mean titer was lower for those nonparalytic cases from whom virus was not isolated Indeed, six had no antibody to any of the three virus types, and nine had none to type 1 The range of titers to the other types in these groups and in normal students was the same * The composite results of both virus isolations and serological studies in 1953 would indicate that even during a severe epidemic a large proportion of cases diagnosed as nonparalytic poliomyelitis are not due to infection with viruses in the poliomyelitis group

The patients excreting virus on whom antibody studies were done all had antibody to the infecting type in their sera, on admission to hospital, and showed only a fourfold average increase in titer

We have investigated only a few contacts of cases but, of 20 family members of 6 paralytic cases, 14 yielded virus of the type infecting the patient Virus titration in stools of contacts was done in only one family, the virus titers being of the same order as the patient's In 56 cases of poliomyelitis, the median titer of virus in the stools was 10[3 *] TC ID_{50} particles per gram of stool

DOCTOR J T SYVERTON (*Department of Bacteriology, University of Minnesota*). These remarks concern the discussion by Doctor Melnick and the paper by Doctor Brown My comments are presented in extension of the discussion by Doctor Tobin I refer you to TABLE 1

* Tobin, J O H K T Brunner, P M Ellwood & J T Syverton 1954 Bull Univ Minn Hosp 26 2[?]4

nesota and neighboring states who were admitted from 1946 to 1954 with a clinical diagnosis of poliomyelitis to hospitals in Minneapolis These viruses are now in storage The specimens utilized for study consisted of feces, throat washings, and brain or cord tissue Viruses were isolated in from 1 to 6 days, commonly in less than 48 hours Isolation and typing can be accomplished as a single step

It can be seen from TABLE 1 that the specimens were representative of poliomyelitis in the North Central states for 1946, 1948, 1949, 1950, 1951, 1952, 1953, and 1954 The percental distribution for 810 strains by immunologic type were. type 1, 77 per cent, type 2, 7 per cent; type 3, 16 per cent.

Two points are worthy of emphasis. (1) the percental distribution by immunologic type for these 810 strains is essentially the same as that found for the 196 strains collected from all parts of the world and typed by the Committee

evidence that the results for any single year, or for several years, should not be accepted as representative of the occurrence of poliomyelitis by virus type For example, the results of 176 isolates for 1954 show a type distribution totally different from that found during any of the preceding eight years It is important that large numbers of cases be studied in consecutive years to provide authoritative information

RELATION OF POLIOMYELITIS VIRUS TYPES TO CLINICAL DISEASE AND GEOGRAPHIC DISTRIBUTION: A PRELIMINARY REPORT

By Alexis Shelokov, Karl Habel, and Donald W. McKinstry
Laboratory of Infectious Diseases, National Microbiological Institute, National Institutes of Health, Bethesda, Md

During the poliomyelitis season of 1952, stool specimens were collected in 22 hospitals in this country and Canada, including areas of high, intermediate, and low attack rates In each area, 10 to 20 poliomyelitis patients contributed specimens in the first two weeks of their disease The only area in which large scale sampling was carried out was Washington, D C, where approximately one half of all the poliomyelitis patients were included The stools were tested in tissue culture by a modification of the techniques described by Enders, Weller, and Robbins,[1] using suspended monkey kidney fragments in flasks, subsequently transferred to monkey testicular tissue in roller tubes for identification and typing In the latter part of the study, the Youngner tech- [illegible] for both primary iso- [illegible] both methods We [illegible] 423 patients, *i e*, an over-all isolation rate of 85 per cent The rate of positive isolations from paralytic patients, however, was 92 5 per cent. Specific clinical information was obtained on the patients in order to confirm the clinical diagnosis

The 1952 poliomyelitis season was characterized by an unusually large number of reported cases throughout this country and Canada The reported incidence rates in the United States by county are shown in FIGURE 1[3] A massive block of counties with consistently high incidence rates of over 40 cases per 100,000 population covers the North Central states, approximately extending from the Great Lakes into the Great Plains area between the Rocky Mountains and the Mississippi River The South Central and the Western states had a scattered occurrence of high rate foci, but the East generally experienced considerably lower attack rates during that year.

In considering the geographic distribution of poliomyelitis virus types in 1952, we have available information on 800 typed isolations from 32 areas In FIGURE 2 we have marked the 20 areas from which specimens were studied by our laboratory, and 14 areas, results from which were available to us by personal communication[4] or from published reports[5] of other workers In 2 of the 14 areas, specimens were studied by us as well as elsewhere It can be seen that fairly good coverage of the country was obtained except for the Far West

Because of the small number of isolations in many of the specific locations, [illegible] ented on the [illegible] astern [illegible] hians [illegible] West [illegible] untry

It is apparent that type 1 viruses were predominant through[illegible]

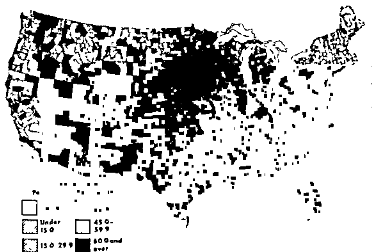


FIGURE 1 Incidence rate of reported poliomyelitis by county 1952

in 1952, although strains of all three types were encountered in every region This predominance of type 1 was particularly evident in the Central and Western regions The percentage of type 1 isolations in the Eastern States was definitely lower, while the percentage of type 2 viruses was relatively higher [illegible] essentially identical with the mean for the country as a whole

Comparing FIGURE 1 and TABLE 1, it is seen that, generally, there was a suggestive correspondence between the areas of high incidence rates and those showing a greater frequency of isolations of type 1 virus

In TABLE 2 we have attempted to compare the virus type distribution with the incidence rates The results from the 32 areas were arranged in order of decreasing magnitude of their reported attack rates[6] The series was then grouped by quartiles each quartile including the results from eight areas The percentage distribution of each virus type within the quartiles indicates that from the areas of highest attack rates type 1 viruses were most frequently isolated while, in the quartile representing the lowest rates, the type 1 predominance was much less marked There is a close agreement between the United States incidence rate of 37 2 cases per 100 000 population and the median incidence rate of 41 5 in our series of 32 areas

In the next three tables dealing with the relation of virus types to the clinical forms of poliomyelitis, we have included only the results obtained in our own study Sufficient clinical information was available on 241 patients from whom *typable* poliomyelitis virus strains had been isolated For the purposes of this

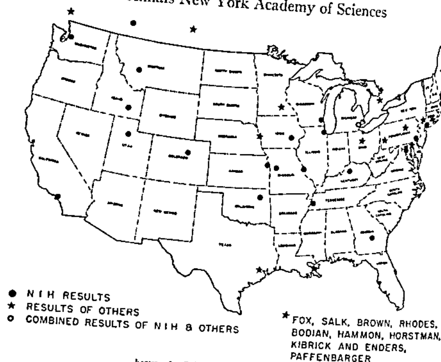


FIGURE 2 Poliomyelitis virus isolations in 1952

TABLE 1
REGIONAL DISTRIBUTION OF VIRUS TYPES, 1952

Region	Number	Virus type (Per cent of isolations)		
		1	2	3
East	227	62	29	9
Central	504	88	8	4
West	63	83	8	9
Total	794	80	14	6
North Central	297	94	5	1
South Central	207	80	13	7

evaluation, a suggestive history and physical findings of fever and meningeal signs, plus positive spinal fluid findings, *i e*, pleocytosis in excess of 10 leucocytes per cu mm and or elevated protein in excess of 45 mgm per 100 cc were required for a presumptive diagnosis of nonparalytic poliomyelitis Recorded clinical evidence of bulbar or spinal involvement was necessary for a case to be considered paralytic

TABLE 3 shows the relative proportion of paralytic and nonparalytic cases tabulated by virus type and by age Age 15 years was selected as the arbitrary dividing line between pediatric and adult cases The striking feature of

TABLE 2

RELATION OF INCIDENCE RATE TO PERCENTAGE DISTRIBUTION OF VIRUS TYPES, 1952

Range of Attack rates* in quartile (cases per 100,000 pop)	Number	Virus type (Per cent of isolations)		
		1	2	3
103–445	270	94	4	2
43–94	156	77	10	13
16–40	235	77	20	3
4–15	133	59	28	13

* Median attack rate in this series—41 5 U S attack rate—37 2

TABLE 3

PERCENTAGE DISTRIBUTION OF PARALYTIC AND NONPARALYTIC CASES BY AGE AND VIRUS TYPE, 1952

Type of disease	Virus type									Total of 3 types		
	1			2			3					
Age in years	No	NP (%)	P (%)	No	NP (%)	P (%)	No	NP (%)	P (%)	No	NP (%)	P (%)
0–14	135	25	75	48	23	77	17	29	71	200	25	75
15+	30	27	73	6	33	67	5	20	80	41	27	73
Total	165	25	75	54	24	76	22	27	73	241	25	75

TABLE 4

PERCENTAGE DISTRIBUTION OF VIRUS TYPES IN BULBAR AND SPINAL PARALYTIC CASES, 1952

	Number	Virus type (Per cent of isolations)		
		1	2	3
Bulbar and bulbo-spinal	46	68	17	15
"Pure" spinal	134	68	25	7
Total of para and nonpara cases	241	68	23	9

this table is the uniformity of the percentage distribution of paralytic and nonparalytic patients within each virus type regardless of age and in spite of the small sample in some of the groups The uniformity of paralytic and nonparalytic patient ratios is even more notable if age groups are combined for each virus type The 3 1 ratio of paralytic to nonparalytic cases in this series is a reflection of the attempt to sample a hospitalized group of patients whose *clinical diagnosis of poliomyelitis was established presumably beyond doubt by* the presence of paralysis

In the next table (TABLE 4), the 180 *paralytic* patients from whom typable poliomyelitis viruses were isolated were divided according to the presumed site

Table 5
Percentage Distribution of Cytopathic Agents from Clinical Polio Cases in Washington, D. C., 1952

	Number	Per cent of isolations			
		Polio virus type			Untypable
		1	2	3	
Paralytic	63	64	33	3	0
Nonparalytic	87	25	10	4	61
NP with neg CSF	5	20	20	0	60

of the lesion as evidenced by the clinical signs of spinal or bulbar involvement As there were only a few patients of clinically "pure" bulbar lesions, they were combined with the bulbo-spinal cases and contrasted with the "pure" spinal group For comparison, virus type distribution for all patients in our series is also given. The close agreement between the relative distributions of the three types within each patient group is apparent in spite of the relatively small number of bulbar cases It is of interest to note that one of the cooperating hospitals supplied us with central nervous tissue material from eight bulbar and bulbo-spinal patients of different ages. We succeeded in isolating type 1 virus from four, type 2 from one, and type 3 from two of these fatal cases, all occurring over a period of a few weeks in the same city

As previously mentioned, our sampling of the Washington, D. C., area was quite adequate Over one half of the cases reported in the entire metropolitan
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"untypables " If the percentage distributions are calculated on the basis only typable polio viruses, they are found to be quite similar in the two groups In an additional group of five patients whose clinical illness was identical with nonparalytic poliomyelitis, except for normal spinal fluid findings, two typable polio viruses and three untypable agents were found. Although only 21 untypable agents were isolated from specimens collected in areas other than Washington, 18 of them also occurred in nonparalytic patients

Thus, within the limitations of the small numbers involved in some areas, the lack of information on the past polio experience of the area, the inadequacy of sampling in certain age groups, and taking into consideration the fact that the data represent the situation during a single poliomyelitis season, the results of this study can be summarized as follows

(1) Type 1 poliomyelitis viruses were predominant throughout the United States in 1952

(2) The predominance of type 1 strains in the North Central states seemed be correlated with relatively high incidence rates. In the Eastern region, here the other two types occurred more frequently, the incidence rates were und to be considerably lower.

(3) In paralytic and nonparalytic patient groups, the distribution of the ree virus types isolated was the same, and this distribution did not appear be influenced by age.

(4) In bulbar and spinal paralytic patient groups, the distribution of the ree types was also similar.

(5) The unusually high proportion of untypable agents isolated from non-ralytic patients in one of the areas studied emphasizes the lack of certainty making a specific diagnosis in this type of illness, even during a "poliomye-is season," without laboratory confirmation.

Acknowledgments

Acknowledgment and appreciation for the collection of specimens is hereby pressed to the following individuals and their institutions. Without their terest and cooperation this study would not have been possible.

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ISOLATION OF VIRUS AND THE DEVELOPMENT OF NEUTRALIZING AND COMPLEMENT-FIXING ANTIBODIES IN POLIOMYELITIC PATIENTS*

By Joseph L. Melnick
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From the data accumulating in a number of different laboratories over the past four years, it appears that poliovirus can be isolated from paralytic patients with a frequency of over 90 per cent, if properly collected specimens are tested in tissue culture, but with less regularity from patients diagnosed nonparalytic poliomyelitis or aseptic meningitis. During sharp outbreaks, the frequency of isolations in the nonparalytic group may be much higher, suggesting that, in such sharp epidemics, poliovirus accounts for more of the aseptic meningitis cases than it does in areas where poliomyelitis rates are low and in areas where there is a clear dichotomy in the epidemic curves of paralytic and of nonparalytic cases. During the summer of 1954, Godenne and Riordan found, among the poliomyelitis patients entering New Haven Hospital, that 93 per cent (36 of 39) of paralytic patients were harboring poliovirus in their intestines as against only 33 per cent (9 of 27) of the nonparalytic patients. None of the paralytic cases yielded an orphan virus, in contrast to 19 per cent (5 of 27) of the nonparalytic cases.

I should now like to add to the remarks of Doctor Shelekov (who discussed the types of poliovirus found in this country) by referring to some data on the global distribution of poliovirus and orphan viruses. Doctor A. M-M. Payne, of the World Health Organization of the United Nations, Geneva, Switzerland, kindly sent me a summary of the isolations made in poliomyelitis laboratories around the world (see TABLE 1). To them I have added the recent isolations made in our laboratory, including those obtained by Doctor Horstmann from her Egyptian infants.

It is of interest to compare the situation in the two neighboring countries of Egypt, where poliomyelitis epidemics have not yet occurred, and Israel, where serious outbreaks have taken place each year since 1950. In 1951, specimens from Egypt were collected for us by Doctor Robert Ward from 36 six-month-old infants. Such young children make excellent sentinels, for at this period they have just lost their maternal antibodies and, like sponges, they sop up viruses from their environment. From the 36 rectal swabbings, we isolated 11 viral agents:[2] 9 Coxsackie viruses, 1 poliovirus, and 1 orphan virus. The

the Middle East. This finding is borne out by Bernkopf's results in Israel: of the 45 viruses which he isolated, 14 were orphans. He obtained a much

* Aided by a grant from the National Foundation for Infantile Paralysis Inc., New York, N. Y. These remarks were prepared by invitation as a discussion to follow the preceding papers of Doctor Gordon C. Brown and of Doctor Alexis I. Shelokov.

TABLE 1
DISTRIBUTION OF VIRUS TYPES ISOLATED IN TISSUE CULTURE

	Type 1	Type 2	Type 3	Orphan
gypt	8	4	4	91
rael	29	1	1	14
outh Africa	82	56	41	3
eden	350	1	1	4
nited Kingdom	45	30	13	
nited States	1250	88	142	80
her countries	148	21	22	33
Totals, 2562	1912	201	224	225
r cent of total polioviruses	82	9	9	
r cent Orphans				9

DISTRIBUTION OF POLIOSTRAINS ISOLATED AND TYPED IN MONKEYS

	Type 1	Type 2	Type 3	
umber	161	20	15	
r cent	82	10	8	

gher percentage of poliovirus isolations which is explained by the fact that s specimens were collected only from patients hospitalized for poliomyelitis ype 1 poliovirus was the chief etiological agent in Israel, as it was in Sweden outh Africa and England are characterized by the high frequency of types 2 d 3 isolations

Of the 2562 strains from around the world which have been isolated and ped in tissue culture, 9 per cent have turned out to be orphan viruses[1] Of e polioviruses, 82 per cent have been type 1, 9 per cent type 2, and 9 per cent pe 3 These figures are remarkably similar to the 82 per cent, 10 per cent d 8 per cent distribution of types 1, 2, and 3 viruses found by the Committee Typing of The National Foundation for Infantile Paralysis, Inc., New York, Y, which typed 196 strains in monkeys

I should now like to comment on the serological response in poliomyelitis octor Brown has presented data which show that patients often enter the spital with neutralizing antibodies already at such high levels that further creases are difficult to measure, a finding with which there seems to be general agreement Such high levels—serum titers of 100 and over—are often und in the normal population and are without diagnostic significance The mplement fixation response is often helpful here. Typical examples of the rological response in patients are shown in TABLE 2.[4] High titers[5] and avidity ores[6] are found only after a recent infection, or for even briefer periods after posure to formalinized vaccine[4] (FIGURE 1).

It is now our practice to use eight units of tissue culture antigen, an amount mparable to that recommended by Svedmyr *et al*,[6] for the test with more ncentrated antigen is more sensitive in picking up small amounts of antibody ith large amounts of antigen, however, the type specificity is usually *lost*,[6] cept with sera of very young children who, presumably, are experiencing eir first poliomyelitic infection A combination of tests for both complement fixation (C F) and neutralizing antibodies on the first specimen has

TABLE 2
SEROLOGICAL RESPONSE IN POLIOMYELITIS, TYPE 1 INFECTIONS

Age	Day after onset	Neutralization			C F titer			C F avidity*		
		1	2	3	1	2	3	1	2	3
6	6	4	<4	<4	<4	<4	8	0	0	8
	14	256	<4	<4	16	16	16	>22	>17	25
8	1	38	<8	<8	32	32	32	>42	38	>42
	28	256	<8	<8	16	16	16	29	25	25
7	12	128	<8	<8	16	4	16	>25	3	>27
	34	128	<8	<8	16	4	16	>32	4	>22
7	12	128	128	2048	16	16	16	>31	16	>31
	36	256	128	4096	16	8	16	>29	10	>29

* See Black and Melnick[4] for method of calculation

proven helpful positive C F tests of high titer are presumptive that a poliovirus is involved, and the neutralization test, if monotypic, suggests the in-

and in only 12 by neutralization There were 15 patients with aseptic menin-

gitis patients, an orphan virus was isolated

Because the C F response in poliomyelitis is so broad, response to any one type may simply reflect exposure to any one of the three types This is clearly seen in the results from the inoculation of 30 children with a formalin-inacti-

C F antibodies did appear, with but three exceptions, they were *only against those types with which there had been previous natural experience*, as indicated by the neutralization tests on sera collected prior to inoculation The presence of type 1 neutralizing antibody had no significant effect on the probability of a type 2 or type 3 C F response after injection of type 1 antigen, and vice versa

Information on the broad C F response is useful to help explain the serological observations recently made in Connecticut and shown in TABLE 5, namely that complement-fixing antibodies can occur in children without detectable neutralizing antibodies against any of the three poliovirus types The frequency with which C F and ...
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higher percentage of poliovirus isolations, which is explained by the fact that his specimens were collected only from patients hospitalized for poliomyelitis Type 1 poliovirus was the chief etiological agent in Israel, as it was in Sweden South Africa and England are characterized by the high frequency of types 2 and 3 isolations

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* See Black and Melnick* for method of calculation

proven helpful positive C F tests of high titer are presumptive that a polio-virus is involved, and the neutralization test, if monotypic, suggests the infecting type A multiple antigen containing all three poliotypes has been used to advantage for such screening purposes The data summarized in TABLE 3

gitis which could be distinguished from nonparalytic poliomyelitis by laboratory studies all but one gave clear negative antibody responses to polioviruses, and one patient had a questionable reaction From six of the aseptic meningitis patients, an orphan virus was isolated

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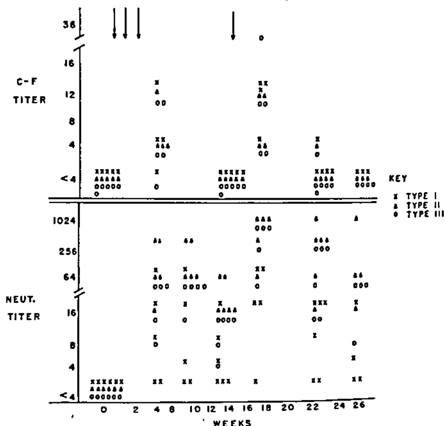


FIGURE 1 Response of six monkeys to inoculation of trivalent formalinized poliovaccine The time of each intramuscular inoculation of one ml of vaccine is indicated by an arrow The neutralizing antibody responses are similar to those reported by Salk and his co-workers Complement fixing antibodies appeared shortly after the first course of injections and then fell to below detectable levels by the 14th week A booster injection at this time was followed again by a sharp but transitory rise of C F antibodies

TABLE 3

CORRELATION OF LABORATORY DIAGNOSIS WITH CLINICAL DISEASE, VIRUS ISOLATION, AND SEROLOGICAL RESPONSE IN 63 PATIENTS DIAGNOSED AS HAVING POLIOMYELITIS

Clinical illness			Laboratory diagnosis type of infection	Virus isolated	Confirmed by		Virus not isolated	Diagnosed by	
Bulbar	Paralytic	Nonpar			Neut	C F		Neut.	C F
5	11	11	Poliovirus 1	27	14	27			
	1	2	1				3†	0	3
		4	2				4	1	4
4	3	2	3	9	2	9			
		5	3				5	2	5
		6	Orphan*	6	6	6			
		9	Unknown but not poliomyelitis*				9	9	8

* Confirmation of the orphan virus and unknown infections as not poliomyelitis indicated by negative reactions to poliovirus in the neutralization and C F tests or by negative C F tests associated with stationary levels of neutralizing antibodies

† No stool samples available on two of these children

TABLE 4

CHANGES IN C F ANTIBODIES IN CHILDREN AFTER INOCULATION WITH TYPE 1 FORMALIN-INACTIVATED VIRUS

Type of antibodies	Neutralizing antibodies prior to inoculation	Number without C F antibodies preinoc	C F antibody response	
			Positive	Negative
1	−	10	1	9
	+	20	8	12
2	−	15	1	14
	+	15	9	6
3	−	13	1	12
	+	13	7	6

TABLE 5

DISTRIBUTION OF C F ANTIBODIES IN 28 NORMAL CHILDREN, AGED 6 TO 9, WITHOUT DETECTABLE NEUTRALIZING ANTIBODIES AGAINST ANY OF THE 3 POLIOVIRUS TYPES

Serum titer	Type 1	Type 2	Type 3	Type 1	Type 2	Type 3	Avidity
16	5	6	23	4	4	18	16–36+
8	3	5	5	3	4	8	8–15
4	5	3	0	6	6	2	1–6
<4	15	14	0	15	14	0	0

TABLE 6

C F REACTIONS IN CHILDREN WITHOUT NEUTRALIZING ANTIBODIES

No of child and date serum obtained	Serum titer			Avidity		
	I	II	III	I	II	III
#218						
4 May 1954	0	0	16	0	0	14
18 June	0	0	8	0	0	10
19 Nov	16	4	16	15	1	>25
#244						
5 May	0	0	8	0	0	7
23 June	0	0	8	0	0	15
3 Nov	4	8	16	3	11	26
#198						
4 May	16	0	16	>29	0	17
18 June	16	8	16	>29	2	19
19 Nov	16	8	16	>31	5	>27

of antibody in the spring and made only C F antibodies during the summer months Each of the neutralizing antibody measurements was carried out in tests which included control sera When present, small amounts of type 3 antibody were readily detected in the test The most attractive interpretation of these data –to us at least – is that a type 4 poliovirus, presumably of low virulence, exists in nature, and produced a heterotypic C F response in the children studied

The available evidence suggests that, like type C influenza virus, type 4 poliovirus should rarely produce disease Because of the group C F. response

among the polioviruses, however, infection with type 4, if it exists, may be detected by the appearance of C.F. antibodies (but not neutralizing antibodies) against any one of the known three types.

The papers of Doctor Brown and Doctor Shelokov, as well as this discussion, have purported to show that, while much new information has recently accumulated in regard to virus isolations and serological responses in poliomyelitis, there are still opportunities for investigative work in this field

References

1 Freyche, M J, A. M-M Payne & C Lederrey. 1955 Poliomyelitis in 1953 Bull [illegible]

4 M[illegible]

5 S[illegible]

6 B[illegible]

POLIOMYELITIS AND THE COMMUNITY

By Alexander D Langmuir
Communicable Disease Center, United States Public Health Service, Atlanta, Ga

In this paper, I propose to extend my remarks to questions pertinent to the concept of the virus and the community.

As a former "shoe leather" epidemiologist who now develops his callouses elsewhere than on his feet, I sit at my desk lost in admiration of the technological advances reported in this monograph, and of their brilliant application to the field problems described More and more, it is becoming apparent that the three separate types of poliomyelitis are behaving in the classical patterns of the familiar contagious diseases This is a fundamental point that will serve as the underlying theme of my discussion

At the conference on which this monograph is based, I sensed a quiet enthusiasm, an optimism that I do not remember at previous conferences on poliomyelitis The reason is obvious Within the past three years, a theory of the pathogenesis of the disease, including the existence of a viremia stage, has become thoroughly established Furthermore, almost certainly within the next two to three years, or sooner, one or more effective immunizing agents *will become generally available to physicians and public health officials* Now is the time for epidemiologists to make the best use of our new understanding and our much anticipated new weapons Let us reappraise our position, reestablish our goals, and prepare for the conquest of poliomyelitis

Doctor Joseph A Bell has stated, and I believe I quote him correctly, that our ultimate aim is "to alter the relationship between the host and the parasite so as to prevent paralytic disease" In my judgment this objective is only a partial one The statement implies that the poliomyelitis viruses could and should remain with us and continue to spread, presumably harmlessly, about the population If the viruses were to remain, almost certainly some individuals would fail to be immunized and at least sporadic cases with paralysis would continue to occur

I believe a sounder goal should be established We should seek not just to alter the host-parasite relationship, but to disrupt it, so that the poliomyelitis viruses may be eliminated, even eradicated from the country Evidence is becoming increasingly strong that this goal is scientifically tenable and reasonably attainable within a few years

The basis for this optimism is the accumulating mass of evidence that the three types of poliomyelitis are behaving, epidemiologically, like the common contagious diseases The mechanism for the survival of these infectious agents is quite well understood It depends upon the balance of immunes and susceptibles *in the population Measles is the classic prototype Under normal* circumstances, epidemics occur only when the proportion of susceptibles exceeds a certain threshold When this proportion of susceptibles is lowered by either natural or acquired immunity, epidemics do not spread

This mechanism has long been respected by epidemiologists as one of the most firmly established forms of the host-parasite relationship. Certainly, all measures designed to reduce the contact rate or the spread of infection from person to person have been unsuccessful and have served only to postpone infection, thus causing some age shift in incidence to older groups. Indeed, many of the acute contagious diseases have continued to exist for centuries with little or no change in incidence. Poliomyelitis is almost certainly one of these.

While the mechanism of survival may be firmly established, it has one vulnerable point, namely, a high level of immunity. If an effective vaccine becomes available and is widely used, then the proportion of susceptibles can be promptly lowered below the level that permits epidemic spread. Endemic spread alone will be insufficient to maintain survival. The infection must disappear.

We have seen this process come slowly for smallpox and more rapidly for diphtheria, very much in relation to the degree of acceptance of the respective vaccines. The process is now complete for smallpox, and is rapidly becoming so in many areas for diphtheria, even though large numbers of the population have never been immunized and even though, in a still larger number of persons, the immunity has waned.

In eradicating poliomyelitis, our problems will be relatively easy. When a safe and effective vaccine is released, very wide acceptance will be immediate, a matter of months. The proportion of susceptibles remaining in the population will be lowered very substantially. Wherever this occurs, epidemics of poliomyelitis cannot occur. A scattering of endemic or sporadic infections will not be sufficient to maintain the chain of infection, and the viruses must disappear. If immunization is widely accepted throughout the country, eradication of the viruses should follow promptly.

It is now most appropriate to endeavor to visualize the problems that will be encountered at that time. Cases will continue to be reported as poliomyelitis, especially the nonparalytic form of the disease. A large number of familiar agents cause such a syndrome, and other agents, less familiar or not yet characterized, certainly exist. An outbreak of one of the arthropod-borne encephalitides, or a group of cases of mumps, encephalitis, pleurodynia, or leptospirosis, if first diagnosed and reported as poliomyelitis, may well lead to community hysteria just as a smallpox outbreak does now.

It is essential, therefore, that a surveillance of poliomyelitis and similar diseases be established throughout the nation by public health authorities, backed by laboratories equipped to isolate and identify not only the poliomyelitis viruses but also the other agents that may simulate poliomyelitis.

If a particular outbreak does not prove to be poliomyelitis, only prompt clinical and epidemiological investigation in the field, backed by competent [illegible] diagnostic [illegible] will allay hysteria. If the outbreak does prove [illegible] ...act of immediate ...en without delay. ...et the eradication

of the poliomyelitis viruses from the country as our logical ultimate goal. If the theory is valid that the disease is caused by an obligate human parasite which depends for survival upon the balance of immunes and susceptibles, then, whenever a safe and effective vaccine becomes generally available, eradication of the viruses from the country should follow promptly.

Part V. Immunization Against Poliomyelitis

POLIOMYELITIS INFECTION IN IMMUNIZED CHIMPANZEES*

By Howard A. Howe

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Circulating antibody is now generally believed to play a dominant, if not an exclusive role in immunity from paralysis in poliomyelitis. The authority for this statement comes largely from experiments on laboratory primates, but there are certain confirmatory observations which have been made on man himself. As yet, these experiments are rather limited, though they are accumulating rapidly at this time. It seems clear that the presence of circulating antibody in the blood serum of adult human beings is connected with their immunity to paralysis, even though these levels in some individuals appear to be very low. The difficulty of investigating a disease like poliomyelitis, in which the incidence of paralysis is very low, has enforced considerable dependence upon laboratory studies. Various experiments have therefore been undertaken in chimpanzees, since it is believed that the immune mechanisms of this animal and of man are very similar, if not identical. For example, both species readily become alimentary virus carriers with a low paralytic rate,[1,2] both show viremia and rapidly mobilize antibody after the stage of viremia,[3] both species react to killed vaccine in a quantitatively similar fashion,[4] and both develop high antibody levels during convalescence from either paralytic or alimentary infection.[5] Avirulent strains of poliomyelitis virus have been fed to human beings with results that are similar to those observed in chimpanzees.[6,7] But the latter species still possesses a certain advantage in that it may be fed highly virulent strains of this virus, and the course of infection and its immune response may be studied throughout in great detail. For example, chimpanzees have been passively immunized by the injection of hyperimmune serum in sufficient quantity to produce serum antibody levels ranging from 1 in 10 to 1 in 100.† Animals showing these levels were fed virulent virus, following which neither paralysis was observed nor could virus be detected in the blood, although both were noted in control animals.[3]

This paper deals primarily with observations on 19 chimpanzees immunized with formol-inactivated poliomyelitis virus. These experiments have been described in greater detail elsewhere.[5] The animals received large doses of vaccine over a considerable period of time in an effort to obtain maximum antibody responses. One group of 9 animals (the F series) received type I vaccine alone, while a second group of 10 animals (the G series) received a vaccine containing all three virus types. At the cessation of immunization the animals were fed active type I (Brunhilde strain) virus and were observed especially for the appearance of paralysis and for the frequency and duration of alimentary infection, as evidenced by virus in the feces. After an observation period of about two years, the animals were again challenged with

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc., New York, N. Y.

† Antibody levels are designated by a figure which, on a log scale, represents that dilution of serum capable of producing a 50 per cent end point with 100 infective doses of virus.

spectively, after the type I challenge.

Following the initial feeding of type I virus, several things were apparent. First, there were no paralyses. This could have been a chance result since, actually, none of the five unvaccinated control animals was paralyzed either. However, the over-all paralytic rate for this particular challenging virus pool in other control chimpanzees had been 2/14 or 15 per cent, thus indicating that it was capable of producing paralysis. Therefore, the occurrence of even one paralytic in the vaccinated group would have shown the ineffectiveness of the immunization.

Second, while all of the controls became alimentary virus carriers, only 10 of the 19 immunized animals had detectable virus in the stools during the second or third weeks following the challenge (stools of the first week were not

fected carried virus in lower titer and for a more limited time than the controls. This can be seen in TABLE 1 by comparing lines 7 and 8 with 11 and 12. Here *are shown the virus isolations from the stools of the same animals tested in two* different ways, by direct intracerebral injection, or by instillation into the nares of monkeys. It is recognized that the intracerebral method is considerably more sensitive than the intranasal method. For example, in 36 stools negative by intranasal assay, it revealed virus in 7 instances. With the intranasal method, only 4 of the 19 animals (21 per cent) were found to be infected during the second week, whereas only 2 (11 per cent) were found in the third week. The same stools tested intracerebrally showed that 7 out of 18 (42 per cent) were positive in the second week, and 5 out of 18 in the third week, thus indicating that a considerable number of the stools had dropped below the level at which virus could be detected by the intranasal method. These are to be compared with the control ratios as determined by both intracerebral and intranasal methods, in which one can see that, during the first week, 100 per cent of the controls carried virus in the stools whereas, during the second week, 62 to 70 per cent were still infected (TABLE 1, lines 7 to 12).

Another point of interest relates to the relationship between the serum antibody titer at the time of challenge and the infection rate as judged by virus excretion, as well as the magnitude of the booster effect produced on serum antibody. It can be seen (TABLE 2) that, in general, the higher the serum antibody at the time of challenge, the lower the rate of alimentary infection. It is also clear that, the higher the antibody level initially, the lower the subsequent antibody response to challenge in relation to it, so that animals experiencing challenge with no antibody showed an average rise of 160-fold (2.2 logs), while animals with initial levels of $10^{-2.0}$ were increased to $10^{-3.5}$. Those showing an initial level of $10^{-2.4}$ rose, on the average, only 0.2 of a log, while those coming to challenge with an extremely high level, $10^{-3.4}$, actually suffered

TABLE 1

RATIOS OF CONTROL AND IMMUNIZED OR CONVALESCENT CHIMPANZEES SHOWING VIRUS IN THE FECES DURING THE SECOND AND THIRD WEEKS AFTER HOMOLOGOUS ORAL CHALLENGE

Some Specimens Assayed by Intranasal and Intracerebral Monkey Inoculation

Experimental category	Infecting pool	Virus in feces (weeks after challenge)					
		2		3		3 & 4	
		Ratio +	Per cent	Ratio +	Per cent +	Ratio +	Per cent +
Both methods							
1 Controls	Brun I and II	8/9	88	6/9	67	8/9	100
2. Controls	Brun III	9/9	100	6/9	67	9/9	100
3. Controls	Brun IV	10/10	100	7/10	70	10/10	100
4. Vacc (1st. challenge)	Brun III	8/19*	42*	4/19*	21*	10/19	53
5 Vacc. (2nd challenge)	Brun IV	2/13	15	0/13	0	2/13	15
6 Convalescents	Brun I and II	2/13	15	0/13	0	2/13	15
Intracerebral method							
7 Controls	Brun IV	10/10	100	7/10	70	10/10	100
8 Vacc (1st challenge)	Brun III	7/18	42	4/18*	22*	9/18	50
9 Vacc (2nd challenge)	Brun IV	2/13	15	0/13	0	2/13	15
Intranasal method							
10 Controls	Brun I and II	7/8	88	5/8	62	7/8	88
11 Controls	Brun III	9/9	100	6/9	67	9/9	100
12. Vacc (1st challenge)	Brun III	4/19	21	2/19	11	4/19	21
13 Vacc (2nd challenge)	Brun IV	1/17	13	0/7	0	1/7	13
14 Convalescents	Brun I and II	2/13	15	0/13	0	2/13	15

* These figures, from the original table,[4] were slightly in error (higher)

a small decline in antibody level during the subsequent month It is of interest to note, however, that, regardless of the level at the time of challenge, after the challenge, the levels tended to be of the same magnitude.

Thirteen of these chimpanzees came to a second type I challenge roughly two years *after the first, and, on this occasion, they behaved in a fashion* similar to that just described. No paralyses were observed either in the test group or in 10 controls without antibody, although the latter all became alimentary carriers and developed high antibody titers (TABLE 2, line 1) In those animals in which the original antibody level was high, averaging $10^{-2.4}$, there was no further increase in titer after the challenge, and none of the animals became alimentary virus carriers In the group of nine animals with lower serum *titers at challenge, namely, an average of* $10^{-1.8}$, *six* animals showed antibody increases of sufficient magnitude to raise the average for the entire group by 0 8 of a log, although only two of these nine animals became a demonstrable though transient virus carrier following this challenge. The animals were indistinguishable from a group convalescent from a previous attack of poliomyelitis but never vaccinated (TABLE 1, lines 1, 3, 5, 6) These findings indicate

TABLE 2

RATIOS OF VACCINATED CHIMPANZEES SHOWING ALIMENTARY INFECTION AND TYPE I ANTIBODY RISE IN RELATION TO TYPE I ANTIBODY LEVEL AT THE TIME OF ORAL CHALLENGE

Prechallenge antibody	First challenge				Second challenge			
	Antibody			Virus in stools	Antibody			Virus in stools
	Ratio showing rise	Average at challenge	Average after challenge		Ratio showing rise	Average at challenge	Average after challenge	
0–trace	5/5	0	2.2	5/5	10/10	0	2.7	10/10
$10^{-1.6}$–$10^{-2.4}$	6/9	2.0	2.5	5/9	6/9	1.8	2.6	2/9
$10^{-2.5}$–$10^{-2.9}$	3/6	2.6	2.8	4/6*	0/4	2.6	2.5	0/4
$10^{-3.0}$ +	0/4	3.4	3.1	1/4				

* This figure, from the original table,[9] was slightly in error (3/6)

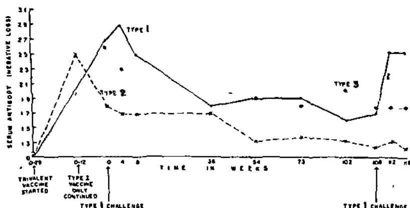


FIGURE 1 Average levels of type 1, 2 and 3 antibody in 10 chimpanzees (G series) vaccinated with formalin inactivated trivalent cord vaccine and challenged orally with type I virus

that, even in convalescents, transient infections may take place if the serum antibody level is below $10^{-3.0}$

Another observation of interest relates to the behavior of antibody over this [illegible]

[illegible]

enced several infections over a period of 91 weeks After the type I challenge

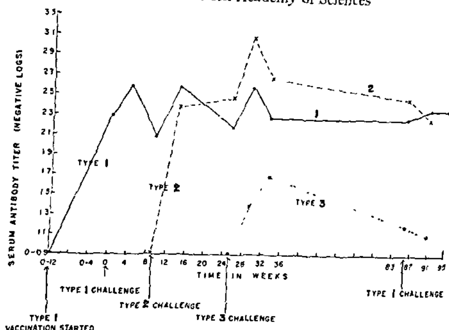


FIGURE 2 Average levels of type 1 2, and 3 antibody in 9 chimpanzees (F series) vaccinated with type I formalin inactivated cord virus and challenged orally with types I, II, and III

same time, the average level of type I in these animals also showed a noticeable *increase, which, however, declined during the next 16 weeks At this time,* these same animals were fed Leon virus (type III) and on this occasion, all
antibody rise to type III
was a very distinct
coincided with the
development of the Leon infection The individual rises were variable in magnitude (0 2 log to 1 7 log) and were not always observed in every animal, but they were sufficient to increase the averages as shown * The increase of type II antibody was particularly striking, and was observed in eight out of the nine animals. It is also significant that the boosting of heterotypic antibody was never observed unless there was evidence that virus infection had taken place Thus, increases in either type I or type II antibody were never observed in the absence of evidence of type III infection, and so on The net result of these repeated infections appears to have been a maintenance of type I and type II antibody at a level very much higher than in the G series of animals (FIGURE 1), so that, at the end of 85 weeks, the average levels were almost as high as they were after the first challenge In consequence of this, when the

* Actual values for individual animals will be found in Howe *

second type I challenge was given, very little evidence of increase in type I antibody level was noted

While similar heterotypic antibody responses have been occasionally noted

in vaccinated animals It would appear, however, that there may very well be antigens in common to all types, and that these are demonstrated more readily in actual infection than in experiments dealing with vaccinated animals only In a series of observations on the challenge of monkeys previously paralyzed by several different strains of type I virus, Bodian[15] noted that up to 65 per cent of the animals in some groups were protected against a subsequent hetero-

Casals, Olitsky, and Brown[16] for mice vaccinated with inactive type II virus and challenged with mouse-adapted type III (Leon) There have also been other suggestions in the conventional typing of viruses[12, 14, 15, 17] that certain rains possess some antigenic relationship to more than one type It is premature to generalize as to the role, if any, that these heterotypic responses ht play in actual immunity It is reasonably clear that, as in the case of ienza strains,[18] the antigenic constitution of poliomyelitis virus strains is variable Hence, the possibility exists that a single encounter with certain strains of poliomyelitis virus may confer some advantage on the individual tions with presumably heterotypic strains

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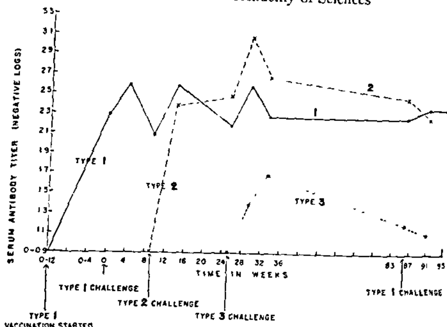


FIGURE 2 Average levels of type 1, 2, and 3 antibody in 9 chimpanzees (F series) vaccinated with type I formalin inactivated cord virus and challenged orally with types I, II, and III

and a small booster effect, the average titers then dropped during the next 8 weeks, at which time, however, the animals were fed type II virus, which infected all of them except one, which had type II antibody at the time of feeding The increase in the average type II antibody level is clearly shown At the same time, the average level of type I in these animals also showed a noticeable increase, which, however, declined during the next 16 weeks At this time, these same animals were fed Leon virus (type III) and, on this occasion, all gave evidence of having been infected The average antibody rise to type III is shown in this slide but it is of further interest that there was a very distinct boost in type II and type I antibodies in the period which coincided with the development of the Leon infection The individual rises were variable in magnitude (0 2 log to 1 7 log) and were not always observed in every animal, but they were sufficient to increase the averages as shown * The increase of type II antibody was particularly striking, and was observed in eight out of the nine animals It is also significant that the boosting of heterotypic antibody was never observed unless there was evidence that virus infection had taken place Thus, increases in either type I or type II antibody were never observed in the absence of evidence of type III infection, and so on The net result of these repeated infections appears to have been a maintenance of type I and type II antibody at a level very much higher than in the G series of animals (FIGURE 1), so that, at the end of 85 weeks, the average levels were almost as high as they were after the first challenge In consequence of this, when the

* Actual values for individual animals will be found in Howe *

second type I challenge was given, very little evidence of increase in type I antibody level was noted.

While similar heterotypic antibody responses have been occasionally noted in human poliomyelitis,[9, 10] their extent and significance cannot be fully appreciated without further work. The three types of poliomyelitis virus were originally separated from one another by methods which were designed to emphasize the antigenic differences between them,[11, 12, 13, 14] the experiments being based upon the failure to demonstrate cross protection or cross neutralization in vaccinated animals. It would appear, however, that there may very well be antigens in common to all types, and that these are demonstrated more readily in actual infection than in experiments dealing with vaccinated animals only. In a series of observations on the challenge of monkeys previously paralyzed by several different strains of type I virus, Bodian[15] noted that up to 65 per

Casals, Olitsky, and Brown[16] for mice vaccinated with inactive type II virus and challenged with mouse-adapted type III (Leon). There have also been other suggestions in the conventional typing of viruses[13, 14, 15, 17] that certain strains possess some antigenic relationship to more than one type. It is premature to generalize as to the role, if any, that these heterotypic responses might play in actual immunity. It is reasonably clear that, as in the case of influenza strains,[18] the antigenic constitution of poliomyelitis virus strains is quite variable. Hence, the possibility exists that a single encounter with certain strains of poliomyelitis virus may confer some advantage on the individual in relation to infections with presumably heterotypic strains.

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Discussion of the Paper

DOCTOR DAVID BODIAN (*Poliomyelitis Laboratory, The Johns Hopkins University, Baltimore, Md*): The very interesting paper by Doctor Howe gives me an opportunity to discuss its bearing on the subject of previous papers on poliomyelitis in the community It may seem inevitable that the attention of so many of us appears to focus primarily on the means of artificial immunization, whether modified or inactivated virus vaccines, rather than on the mechanism of immunization in relation to population immunity. The mechanism of such immunity is still a fertile field of study, especially since there is already much information, and since there are powerful new tools for further investigations

Poliomyelitis is a disease spread by human agencies. If we immunize a person, we then not only may prevent his becoming a patient, but conceivably we may prevent him from becoming an agency for the spread of the disease to others What do we know about this subject? As far as smallpox is concerned, as Doctor Langmuir pointed out in his discussion, we know that in this country immunization has essentially eliminated that disease, although many persons in some of our communities have never been vaccinated These nonimmune individuals do not have smallpox because there is no virus left to infect them Is this going to be the story with poliomyelitis?

This question can be approached from what is already known from experimental work. In experimental animals, serum antibody produced by passive or active immunization can prevent paralytic poliomyelitis What can it do by way of preventing multiplication of virus in tissues that excrete the virus into the environment? We know, as a result of studies in chimpanzees and in human beings, that high levels of serum antibody are present in the preparalytic period of natural infections or of infections by virus feeding[1,2] Yet virus is excreted in high titer in the feces for two weeks or more following onset of paralysis. This proves conclusively that high levels of serum antibody are quite ineffective in preventing viral multiplication in the cells in the alimentary

tract which produce fecal virus, once virus has invaded these cells In other words, serum antibody developed after virus exposure of the alimentary tract, or of the nervous system, does not materially affect viral multiplication in either of these two tissues.

Now it is also known that previous infection of chimpanzees or of human [illegible]

is unable to do so Or, is it possible that prior infection inhibits reinfection of the alimentary tract by virtue of a local persistence of noninfective virus particles remaining from the first infection, or by means of some other local immune process distinct from the effects of circulating antibody?

The latter view seemed more plausible to me until Doctor Howe's experiments were made, since I had shown that high levels of passive antibody given to chimpanzees just before virus feeding did not prevent or inhibit the alimentary infection In retrospect, I believe it possible, however, that my chimpanzees were exposed to virus before the high levels of passive serum antibody (over 1 100) could permeate into tissues of the alimentary tract in which virus multiplies and from which it is excreted Doctor Howe's chimpanzees are interesting and important, first, because the antibody produced by immunization with killed-virus vaccine was present long enough before virus feeding to allow for distribution of antibody into available tissue spaces and, second, because the antigen, being inactivated, could not produce a form of local immunity in the alimentary tract by multiplication prior to the challenge virus feeding

There are certain important points to keep in mind, however First of all, neither previous alimentary infection nor previous vaccination produces complete resistance to alimentary infection with virus of the same type Second, Doctor Howe's work suggests that only animals with high levels of serum antibody produced by vaccination with inactivated virus show any appreciable inhibition of alimentary infection The effective levels are comparable not to the primary response of children vaccinated in Doctor Salk's studies but, possibly, to the levels of the booster response which he reported last summer [8] It is possible, however, that even low serum antibody levels, by preventing viremia, could inhibit fecal virus excretion from foci in the alimentary tract, which may be infected from the blood stream

future mass vaccination Looking at the problem from the point of view of the survival of the virus species, it appears conceivable that, if mass immunization of a population included a large proportion of adults as well as children, even with inactivated virus vaccination plus booster doses, the amount of virus dissemination, from throat or feces, could become so reduced that the virus would disappear from large communities on a continental scale We know

that the survival of poliomyelitis virus populations in smaller human communities may be so precarious, even without immunization, that the virus may disappear for many years, until reintroduced from the outside.

The question is, can this process be assisted in a large community by mass immunization? I believe the answer depends, as is so often the case, on quantitative relations between the potency of vaccines, the degree of acceptance of vaccination, and sociological factors, such as play an important role in deciding the immune status of nonvaccinated populations Moreover, the prevention of reintroduction of the viruses of poliomyelitis into a community appears to be a much more formidable problem than the analogous problem in relation to smallpox.

On the other hand, if it is true that widespread vaccination with inactivated vaccines could conceivably reduce the amount of dissemination of virus in the population by fecal routes of contamination, as well as possibly reduce paralytic [illegible]

of what we know already that, first of all, the populations in which epidemics of paralytic disease are occurring are precisely those populations which already are presumably showing the effects of reduced prevalence and dissemination of excreted virus, so that they could hardly be said to be deprived of widespread virus exposure because of artificial immunization of any kind. It may be said, of course, that artificial immunization by virus feeding with avirulent strains could give such population not only a primary immunological stimulus, but also could produce, to some extent, the spread of excreted virus which would infect and immunize nonvaccinated individuals, or reimmunize those whose immunity has waned after a previous vaccination experience This hope however, seems to be a rather contradictory one, since it is already apparent that populations which would require immunization programs are able to reduce greatly the amount of virus in the environment because of developments in sanitation and other habits of life Populations which scarcely need vaccination, because of a low incidence of paralysis, already have an abundance of poliomyelitis strains in the community which are actively immunizing children soon after they are born

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A CONCEPT OF THE MECHANISM OF IMMUNITY FOR PREVENTING PARALYSIS IN POLIOMYELITIS*

By Jonas E. Salk

Virus Research Laboratory, School of Medicine, University of Pittsburgh, Pittsburgh, Pa.

The principles of immunization, as they appear to apply to the problem of poliomyelitis, have been discussed previously. It is the purpose of this communication to elaborate upon a concept of the dynamics of immunity to paralysis as it has been deduced from certain epidemiologic observations and from studies with noninfectious vaccines. By way of summary, the essential features of the immunologic reaction, in man, following an injection of formaldehyde-treated poliomyelitis virus vaccine, are shown in FIGURES 1, 2, and 3. FIGURE 1 illustrates the level of the antibody response elicited by three doses of vaccine given in a five-week period with the second dose having been administered two weeks after the first. A distinction between the primary vaccination effect, and the secondary, or booster, effect is seen in the comparison of the left-hand and the right-hand frames in FIGURE 1. Persons who have demonstrable antibody from a previous natural infection respond with higher levels of antibody than do those who give no evidence of having had a prior immunologic experience with the respective virus types.

In studies with poliomyelitis vaccines, the same phenomenon has been observed as with other vaccines, where a two-stage procedure is required to raise the concentration of serum-antibody to the very high levels that can be achieved. It has been observed also that a relatively long interval is required between primary and secondary stimulation before the full booster effect can be elicited in man. The minimal interval in man appears to be a number of months. In monkeys, the effect seems to be fully developed in about four weeks. In the mouse, the optimal interval can be measured in days. The relative ineffectiveness of multiple inoculations, when the several doses are given at intervals too short to produce a maximal effect, is illustrated in FIGURE 2. If, however, primary immunization had been adequate, and if a sufficient interval had elapsed between primary and secondary stimulation, then merely a single dose appears to be sufficient to produce a maximal response, as is shown in FIGURE 3.

Present evidence indicates that antibody induced with a noninfectious antigen is not evanescent and, also, that the effects of primary immunization are such that subsequent revaccination, at intervals of up to at least two years, results in sharp rises of antibody to heights comparable to, or beyond, those observed in persons who have had a natural infection. Thus it would appear that man reacts to the poliomyelitis antigen in accordance with the same laws that appear to govern the reaction to other antigens.

The Significance of the Immunologic Complexity of the Poliomyelitis Viruses

In the past several years, many investigators have reported evidences suggesting the existence, in viruses of one type, of antigenic components that

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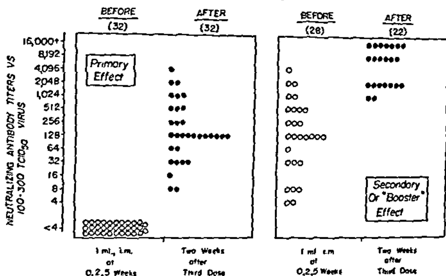


FIGURE 1 Distribution of types 1, 2, and 3 antibody titers before and after vaccination with aqueous trivalent poliomyelitis vaccines Persons with no demonstrable antibody before vaccination (left), and persons with some antibody from previous nonparalytic infection (right)

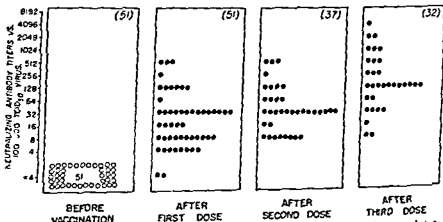


FIGURE 2 Primary vaccination effect Influence upon antibody level of each of three doses spaced at intervals of two and five weeks after the first dose

characterize the predominant antigen of one or both of the other two types Further evidence for this has been obtained in the course of our studies in man. These observations have furnished clues to the probable mechanism whereby, under natural circumstances, paralytic poliomyelitis is controlled by immunologic means I should like to summarize the essential features of the observations that have been made as they bear on this question.

First, I should like to show the extent to which the immunologic response is different in persons who have no antibody to any of the three types prior to vaccination, as compared with individuals who possess antibody for one or

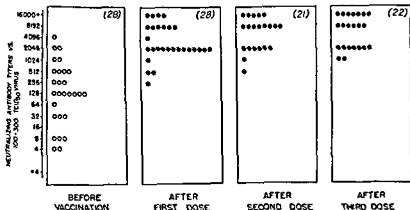


FIGURE 3. Secondary or "booster" effects in persons who have had a natural infection. Influence upon antibody level of each of three doses spaced at intervals of two and five weeks after the first dose.

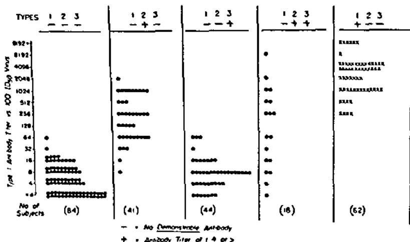


FIGURE 4. Type 1 antibody response to first dose of vaccine in persons with different antibody patterns of prevaccination.

more of the three known types. A comparison of the different degrees of response to the type 1 component of the vaccine, as observed in groups of such persons, all of whom, prior to vaccination, had no demonstrable type 1 antibody, is shown in FIGURE 4. It is to be emphasized that the antibody responses here recorded are those observed after the *first* dose of vaccine. It would appear from these data that persons who had a previous type 2 infection (as indicated by the presence in their blood serum, at the time of vaccination, of antibody for the type 2 virus) responded with much higher levels than did persons who had no antibody to any of the three types. Persons who had a

prior type 3 infection did not react to the same degree as did those who had a prior type 2 infection. The group with type 3 antibody before vaccination reacted not too differently from individuals who had no previous immunologic experience whatever Those who, prior to vaccination, had antibody for both types 2 and 3 virus seemed to react as if they had either a prior type 2 or type

It would seem from the data in FIGURE 4 that the naturally occurring type 2 viruses that were the cause of the infection in these individuals must have possessed a sufficient amount of type 1 antigen to have produced the degree of immunologic hyperreactivity that was revealed by the effect of vaccination Infection with the type 3 viruses, on the other hand, seemed to have produced a barely perceptible sensitizing effect.

When a comparison is made of the response to the first dose of vaccine in persons who do, or do not, possess type 1 antibody before vaccination, one can recognize four grades of reactivity, depending upon the nature of the previous immunologic experience. These observations suggest that the different influences probably depend upon quantitative factors—*i.e.*, the amount of type 1 antigen involved in the previous immunologic experience

Similar data on the type 2 antibody response, in persons with different previous immunologic experiences, are summarized in FIGURE 5. From the degree *of antibody response to the first dose of vaccine in persons* with no prevaccination antibody to any of the three types, as compared with the response among those who had a prior type 1 infection, it is clear that the earlier type 1 experience had induced a significant degree of heightened reactivity to the type 2 component of the vaccine This suggests that the naturally occurring type 1

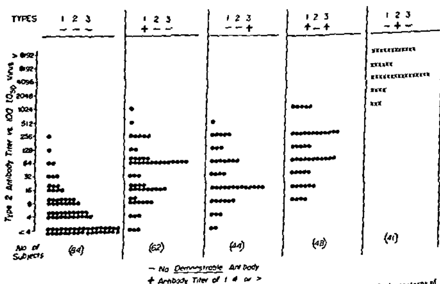


FIGURE 5 Type 2 antibody response to first dose of vaccine in persons with different antibody patterns of prevaccination

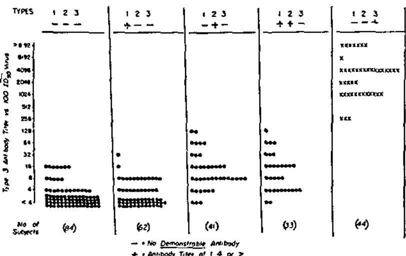


FIGURE 6. Type 3 antibody response to first dose of vaccine in persons with different antibody patterns of prevaccination

viruses must have possessed some of the antigenic configurations that distinguish the type 2 virus. A comparison with the data in FIGURE 4 suggests that a prior type 2 infection may have exerted a somewhat more marked effect on type 1 responses as compared with the effect of type 1 infection upon type 2 vaccine response. In contrast to the absence of any influence of a previous type 3 infection upon the subsequent type 1 response (FIGURE 4), there is observed in FIGURE 5 a significant influence of the prior type 3 infection on type 2 responsiveness. In terms of degree, it would appear that the influence of the prior type 3 infection was only slightly less than was the effect of the prior type 1 infection. The presence of antibody for both type 1 and type 3 viruses seems to have caused a heightened reactivity to type 2 equal to, or greater than, the effect of either a type 2 or type 3 infection alone.

FIGURE 6 illustrates the type 3 response and, since the type 3 component of the vaccine employed in this particular study was less potent than the types 1 and 2 components, the height of the primary response reflects this difference. The amount of antigen present, however, was still sufficient to induce the sharp booster effect in persons who possessed type 3 antibody from a natural infection. The absence of any appreciable effect, of a previous type 1 infection, on type 3 reactivity is clearly evident. A previous type 2 infection, however, did measurably influence type 3 responsiveness.

The relationships observed in FIGURES 4, 5, and 6 are summarized in FIGURES 7 and 8. They indicate that there is little sharing of specific antigenic configurations in the constituents of types 1 and 3 virus, but there must be a substantial amount of type 1 antigen in the type 2 virus, and a definite, but lesser amount, of the type 2 antigen in the type 1 virus. The quantity of type 2 antigen in the type 3 viruses, and of type 3 antigen in the type 2 viruses, is

TYPE 1 RESPONSE | TYPE 2 RESPONSE | TYPE 3 RESPONSE

ANTIBODY LEVEL VS 100 ID_{50} VIRUS

8192, 4096, 2048, 1024, 512, 256, 128, 64, 32, 16, 8, 4, 2

TYPE 1
TYPE 2
TYPE 3

+ = Presence of demonstrable pre-vaccination antibody

FIGURE 7 Geometric mean of antibody response to first dose of vaccine

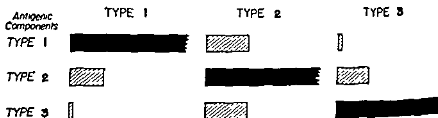


FIGURE 8 Data derived from vaccination studies Interpretation suggesting the existence of major and minor antigens in strains of naturally occurring poliomyelitis virus

similar, but it appears that the amounts are less than the amount of type 1 antigen present in the type 2 virus

The Significance of the Relative Frequency of Paralytic Infections Due to the Three Virus Types

The foregoing observations led to a further inquiry into the possible role of the occult immunologic effects of a prior infection upon the outcome of a subsequent encounter with a heterotypic poliomyelitis virus under natural circumstances The search for an answer to this question was made through analyses of data obtained in the course of other studies

The purpose of the other studies to which I refer was to determine the frequency with which types 1, 2, and 3 infections occur under natural circum-

stances, and then to determine the relative frequency with which each virus type causes paralysis. The way in which the frequency of infection of each virus type was established was by means of antibody studies both in nonparalyzed and in paralyzed persons. The paralyzed population consisted of approximately 560 patients, at the Georgia Warm Springs Foundation. Since these patients derived from a wide geographic area and represent paralytic experiences over a number of years (1920–1954), an effort was made to include in the nonparalyzed population individuals of different ages and from various geographic areas, so as to approximate the over-all experience of the paralyzed population with respect to exposure to poliomyelitis viruses.

Another purpose of the studies in paralyzed convalescents was to determine, by serologic means, the frequency with which infection with the different types had occurred in previous years, to see if some retrospective examination could be made of the prevalent immunologic type in different years and in different parts of the country. This could be done, of course, if antibody levels tended to persist following a paralytic infection, as was found to be the case, and would also be possible if a sufficient proportion of individuals among the paralyzed group were found who possessed antibody for but a single type. In the group of approximately 560 paralyzed patients at the Georgia Warm Springs Foundation, it was found that almost 50 per cent had antibody for only one virus type. There was none who did not possess antibody for at least one type. This finding would suggest that paralytic poliomyelitis of such severity as to require treatment at the Georgia Warm Springs Foundation has been caused, in recent years, by one of the three known types of poliomyelitis virus. This is shown in TABLE 1, where a comparison is made, between paralyzed and nonparalyzed populations, of the number of persons who possess no antibody for

with no antibody to any of the three types whereas, in the paralyzed group, there were no such individuals.

A further comparison of these two populations is made in terms of the frequency with which antibody for types 1, 2, or 3 poliomyelitis virus alone is found among those with antibody for a single type. The results are illustrated in FIGURE 9, where it may be seen that, of every 100 nonparalyzed individuals

TABLE 1

FREQUENCY OF OCCURRENCE OF SERUM ANTIBODY FOR ONE OR MORE TYPES OF POLIOMYELITIS VIRUS

Demonstrable antibody for	Nonparalyzed (1,003)		Paralyzed (563)	
	No.	%	No.	%
Three types	124	12.4	124	22.0
Two types	244	24.3	174	30.9
One type	369	36.8	265	47.1
None	266	26.5	0	0

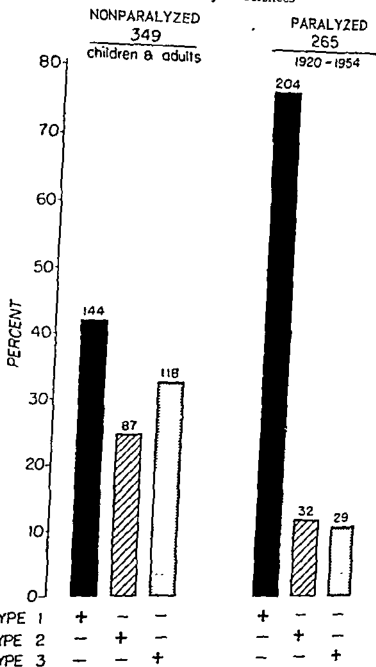


FIGURE 9. Relative frequency of occurrence of antibody for only one type of poliomyelitis virus in nonparalyzed and paralyzed individuals.

who possess antibody only for a single type, 42 have type 1 antibody only, 25 have type 2 antibody only, and 33 have type 3 antibody only. This is in striking contrast to the far greater predominance of persons with only type 1

persons is about the same as is the frequency with which the respective types of virus have been isolated from paralytic cases that have occurred in the past several years The fact that paralysis due to the type 1 virus occurs about seven times more frequently than does paralysis due to types 2 or 3 seems to be

Data on the frequency of occurrence of antibody for two virus types, in different combinations, among nonparalyzed and paralyzed persons is shown in FIGURE 10 It is clear that in both the nonparalyzed and in the paralyzed, *combinations of types 1 + 3 antibody occurred more frequently than did* combinations of types 1 + 2 or combinations of types 2 + 3 The combination 1 + 3, however, occurred more frequently in the paralyzed group than in the nonparalyzed, while the combination 1 + 2 and 2 + 3 occurred less frequently in the paralyzed than in the nonparalyzed The greater frequency, in the paralyzed group, of individuals who have only type 1 antibody suggests that the type 1 virus is most likely to have been the reason for paralysis when the first encounter was with this virus It is conceivable, therefore, that when the type 1 virus is one of the two viruses for which antibody was present, it, too, was the more likely cause of paralysis That this is merely a statement of *probability* and not *always* true is, of course, indicated by the fact that some individuals, within the paralyzed population, have type 2 or 3 antibody only, or the combination 2 + 3 From these comparisons of the frequency of occurrence of the combinations of antibody for types 1 + 3, 1 + 2, and 2 + 3 in paralyzed and nonparalyzed individuals, it would seem that a prior type 2 infection must have a different meaning than does a prior type 3 infection, in terms of whether or not paralysis will ensue upon subsequent contact with the type 1 virus From these data, it would appear that the experience of the previous type 2 infection reduced the probability that the subsequent type 1 infection would be a paralyzing experience or, alternatively, that the prior type 3 infection did not influence significantly or if it did so, certainly to a much less degree the probability of paralysis ensuing upon subsequent contact with the type 1 virus

An analysis in further support of this interpretation is presented in TABLE 2, which shows that the probability of experiencing a nonparalytic infection due to type 1, 2 or 3 virus is essentially the same upon first or second exposure to a poliomyelitis virus This is not the case in paralyzed individuals

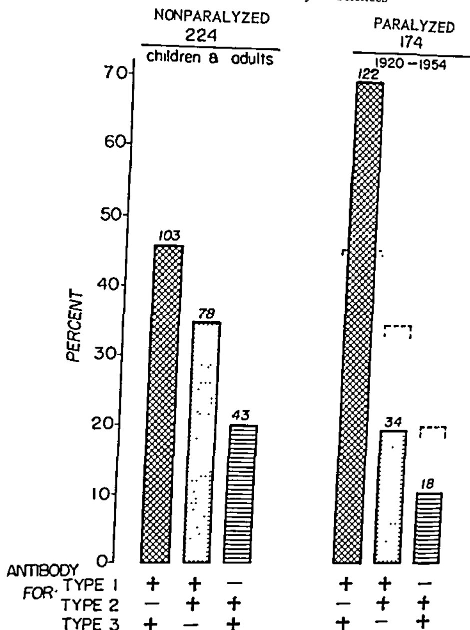


FIGURE 10 Relative frequency of occurrence of combinations of antibody for two types of poliomyelitis virus in nonparalyzed and paralyzed individuals

A Concept of the Immune Mechanism in Poliomyelitis

If the observations presented in the two previous sections of this paper are linked in a relationship of cause and effect, then these two observations—namely (1) the evidence for the sensitizing effect of a prior type 2 infection,

TABLE 2

FREQUENCY OF OCCURRENCE OF SERUM ANTIBODY FOR EACH TYPE OF POLIOMYELITIS VIRUS

Demonstrable antibody for	Nonparalyzed			Paralyzed		
	1	2	3	1	2	3
One type n p —349 p —331	42%	25%	33%	80%	10%	10%
Two types n p —448 p —380	40%	27%	33%	45%	15%	40%

N p —nonparalytic
p —paralytic

as revealed by the response to the type 1 virus in a killed vaccine, and (2) the apparent influence of a prior type 2 infection in reducing the probability of the occurrence of paralysis upon subsequent contact with a type 1 virus—make it appear that the presence of a demonstrable level of circulating antibody for the type 1 virus was not necessarily required for the prevention of paralysis It would appear that paralysis may have been prevented because of the existence of a hyperreactive immunologic mechanism engendered by the type 1 antigen present in the type 2 virus that had caused the first infection If this be so, then it would appear that *the* prerequisite for persistence of immunity may not necessarily be the continued presence of antibody in the blood stream but rather a persistence of the state of immunologic hyperreactivity

The observations presented bear not only on the practical problem of immunization of man against this disease but on many aspects of the theoretical question of the dynamics of the immune process If we understood fully the dynamics of immunity in this disease then, theoretically, we could, by artificial means, reproduce the sequence of events that will lead to the immune state

Many assumptions have been made in support of the selection of one approach or another to the solution of the practical problem Those who think that only a live-virus vaccine will produce the necessary changes required for lifelong immunity must believe that the infectious process contributes something that cannot be conferred by any other form of antigenic stimulation Then there are those who, whether they think that a live- or a killed-virus vaccine is necessary, are of the opinion that the actual presence of antibody in the circulating blood provides the *sine qua non* for effective immunity It would follow that the required level of antibody in the blood would depend upon whether pathogenesis is *via* the blood stream or along nerve pathways If the latter, then a higher level of antibody would be required than if the virus is always transmitted to the central nervous system (CNS) *via* the blood stream But then the possibility must be considered that the blocking effect against virus invasion of the CNS could conceivably take place before entrance of virus into the blood stream, and that this could be effected by a hyperreactive immunologic state which would be triggered by virus as it multiplies at the primary site of infection and, thereby, result in the prompt production of immune substances If the hyperreactive state is adequate to produce a

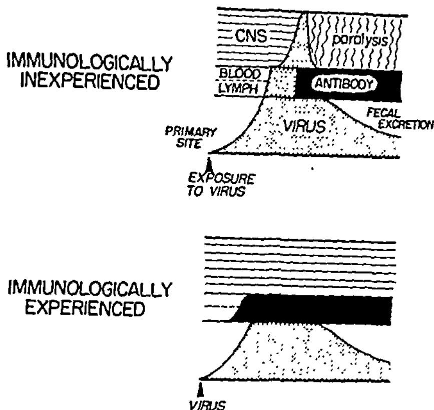


FIGURE 11 Concept of the influence of previous immunologic experience in the prevention of paralysis

sufficient concentration of specific antibody, and if this effect would occur prior to the end of the multiplication period when the virus might normally enter the blood stream, then one can visualize a situation in which a state of immunity would exist even though there is no demonstrable antibody in the circulating blood at the time of virus exposure (FIGURE 11). Such immunity, however, would be dependent upon the capacity of the organism to respond to infection with the immediate production of antibody, sufficient either to prevent further multiplication, or to reduce the length of the period of active infection. The quantitative relationship between the rate of virus multiplication and the rate of antibody formation would determine the extent to which this barrier was effective A highly reactive immune mechanism might be able to block the most actively multiplying virus, whereas a less reactive immune mechanism could conceivably be ineffective Therefore, we must consider the *quantitative* concept of level of antibody required to prevent central nervous system invasion, and the *quantitative* concept as applied to measurements of degree of sensitization that would be required for immunity to be effective even in the absence of a measurable amount of circulating antibody

The data presented suggest that a naturally acquired type 2 infection sensitizes the immune mechanism to a subsequent contact with a type 1 virus It

TABLE 3
SEROLOGIC RESPONSE TO NATURAL INFECTION IN VACCINATED CHILDREN

Subject No	Age	Prevaccination May 4 1954			10 Days after 3rd dose June 18, 1954			5 Months later Nov 19, 1954		
		Type 1			Type 2			Type 3		
		May	June	Nov	May	June	Nov	May	June	Nov
C-7930	5	0	16	8	0	128	8192+	0	32	4
C-7948	7	0	16	16	0	128	8192+	0	1024	128
C 8033	12	0	16	8	0	8	8192+	0	128	32
C 7983	8	0	4	1024	0	4	32	0	128	32
C-7957	6	0	32	2048	0	32	128	256	8192+	8192+

Vaccinated on May 4, 1954, blood tested 10 days after third dose, June 18 and five months later November 19

TABLE 4
TWO-YEAR FOLLOW UP IN FIRST GROUP OF SUBJECTS IN WHOM VACCINATION STUDIES WERE UNDERTAKEN

Age at time of first vacc	Identification No	Type 2 antibody titer vs. 100 ID50 virus			
		Before vacc *	2 Mos	Booster at 20 mos †	22 Mos
years					
2	W 44	0	32	8	2048
4	W 31	0	64	8	1024
5	W 18	0	16	8	512
6	W 27	0	8	2	512
8	W 20	0	128	64	256
9	W 33	0	8	0	256
10	W 8	0	4	0	512
10	W 26	0	16	8	256
11	W 74	0	32	4	512
14	W 34	0	16	8	256
17	W 1	0	32	16	128
17	W 32	0	32	16	N T ‡
31	W 3	0	16	8	256

* 2 Doses I.D. of 0.1 ml each 6 weeks apart monovalent MEF1 1 250 ID50 [illegible]
† 1 Dose I.M. of 1 ml trivalent vaccine
‡ Not tested

would follow from this that the amount of type 1 antigen required to produce the sensitizing effect must be very small indeed Since a measurable amount of type 1 antibody either does not develop, or does not persist for any length of time after a type 2 infection, it would follow that the resistance to the development of type 1 paralysis, which is engendered by the prior type 2 infection is mediated by some means other than type 1 antibody present in the circulating blood *at the time of exposure* to the type 1 virus On the assumption that antibody is the agent that effects immunity, this must mean, then, that type 1 antibody is produced sufficiently rapidly after exposure, and in sufficient concentration, to exert a blocking effect upon virus produced at the primary site of infection

According to this concept, a critical index of immunity would be provided by

a test for the presence of the hyperreactive state rather than by a test that would determine the presence or absence of antibody in the serum. Even if an individual does not possess circulating antibody, he might well be immune [illegible]

vaccination. If the hyperreactive state persists either for long periods, or throughout life, then it is conceivable that immunity to the development of paralytic poliomyelitis would persist similarly (TABLES 3 and 4).

Although the presence of unequivocal concentrations of antibody in the circulating blood is a good indicator of the existence of a hyperreactive immunologic state (TABLE 4), it is suggested that the hyperreactive mechanism, even in the absence of demonstrable circulating antibody, is the minimum requirement for effective immunity. For this reason, tests are being devised to measure degree of immunologic hyperreactivity. Such a test could be based upon the booster phenomenon, since the persistence of the hyperreactive state is reflected in the booster response. Thus, the booster phenomenon would be useful not only to reinforce the level of circulating antibody, but as a test that would reflect the degree of persistence of immunologic hyperreactivity.

Discussion of the Paper

[illegible]

diation[1] instead of formalin. I have been asked to present recent results obtained by our group* in the immunization of children in Morgan County, Illinois, with an aqueous trivalent tissue culture poliomyelitis vaccine inactivated by ultraviolet irradiation. The vaccine used in these studies was prepared as described previously,[1] but no preservative was added because we found that the irradiated vaccine containing no preservative still showed antigenicity in animal potency tests after six months' storage in the refrigerator. The strains of virus selected to represent the three types of poliomyelitis were the same as those used previously, except that the Brunhilde strain was substituted for the Mahoney strain for type 1. The MEF₁ strain was used for type 2, and the Saukett strain for type 3.

One hundred and twenty normal children ranging in age from two to seven years were given three injections of the irradiated vaccine during the late winter and spring of 1954. All of the children were bled prior to inoculation and received two injections intramuscularly one week apart. The third inoculation was given three to four weeks after the second. All the children were bled two weeks after the third inoculation. Antibody titrations were carried out in roller-tube cultures of trypsinated monkey kidney tissue cells. Since we were interested in determining whether primary immunization could be stimulated by the irradiated vaccine, all prevaccination serum antibody titra-

* Including the late Doctor S. O. Levinson of the Michael Reese Research Foundation, Chicago, Ill.; Doctor H. J. Shaughnessy, Doctor Ruth Church, and Doctor Leonard Schuman of the Illinois Department of Public Health; Doctor A. M. Wolf, Doctor F. Oppenheimer, and Martha Janota of the Michael Reese Research Foundation.

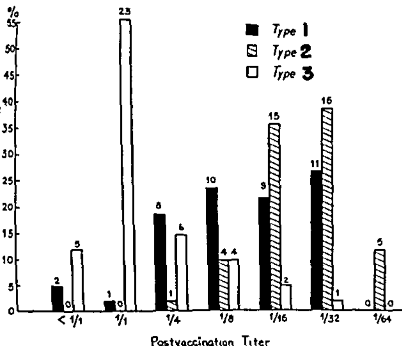


Figure 1. Antibody response to three types of poliomyelitis virus in 41 children with no detectable polio antibody prior to vaccination with aqueous poliomyelitis trivalent tissue culture irradiated vaccine

tion were carried out initially with 10 ID_{50} of each of the 3 virus type components in neutralization tests with undiluted serum (1:1), including serial twofold dilutions starting with 1:4. It was found that 41 children had no detectable [illegible] titer of 1:1 or greater to 10 ID_{50} to one or more of the 3 virus type components subsequently developed a significant (fourfold or greater) antibody response in their postvaccination serums to 100 ID_{50} of the 3 virus types.

No significant local or generalized reactions were encountered in the 120 children vaccinated with the irradiated vaccine. Urinalysis of all of the children was normal when tested at the time of the last blood specimen. Furthermore, we were unable to detect Rh or Hr antibodies or sensitization in 32 Rh negative children in our series of immunized children.

We have found a correlation between antibody response to the irradiated vaccine in man, the mouse,[2] the monkey, and the rabbit.[3] More recently, we have prepared an improved irradiated vaccine from tissue culture virus sus-

pensions with ID_{50} titers of 10^{-7} to 10^{-8} compared to earlier relatively low titered preparations and have also made modifications in the technique of inactivation These improvements in vaccine preparation are reflected in superior antibody responses that have been obtained recently in both immunized mice and monkeys.

References

1. M[illegible]

2 M[illegible]

3 L[illegible]

IMMUNIZATION OF MAN AGAINST POLIOMYELITIS WITH ATTENUATED PREPARATIONS OF LIVING VIRUS

By Hilary Koprowski

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There could hardly be a more opportune time for retrospective analysis of live virus immunization against poliomyelitis. This is the fifth anniversary of the day when—after painstaking soul searching—a decision was reached to administer the TN strain of poliomyelitis in live form to a nonimmune individual. This first recipient, who ingested log of 5.8 PD_{50} of the virus in the form of cotton rat CNS, developed intestinal carriage, followed by the appearance of homologous antibodies.[1] This response, occurring in the absence of any signs or symptoms of illness, spurred a collective effort to continue the study—an endeavor considered, at that time, not only perilous for the subjects of the trial but also as sinful on the part of the investigators, who risked misunderstanding and even downright mistrust by laymen and scientists alike. Five years have not yet changed the Age of Doubt into the Age of Faith, but the administration of live poliomyelitis virus to human subjects is now considered only a minor sin and an [illegible]

sin

"G

clinical trials with particular emphasis on a comparison of data obtained in the oral administration of types 1 and 2, in the second part, the interference phenomenon between types 1 and 2 in the human alimentary tract will be described, and the rest of the paper deals with speculations concerning criteria of attenuation.

Summary of the Trials

In the course of the five years, 87 individuals who had no antibodies against type 2 virus ingested the TN strain.[1, 2] Fifteen subjects, again antibody-free, received type 1 virus, SM strain,[3] relatively recently. The TN strain was administered in the form of CNS suspension of either cotton rat or mouse origin; the SM strain inoculum consisted, in most cases, of a chick embryo tissue culture suspension. Eight of the subjects received gamma globulin before virus administration.

The virus concentration of the inocula was similar for both strains, yet the ratio of virus carriers was entirely different. As shown in TABLE 1, only 54 per cent of the individuals who ingested type 2 virus excreted the agent in their stools. In marked contrast, as will be shown later, all subjects who received type 1 virus became intestinal carriers, excreting virus almost every single day after administration for a long period of time (see below). Type 2 virus was isolated less frequently and disappeared from the intestinal tract 23 days, at the latest, after consumption. With a single exception, every individual who was fed either type of virus developed homologous antibody.

TABLE 1
RELATION BETWEEN TYPE OF VIRUS INGESTED, CARRIER STATE AND ANTIBODY RESPONSE IN MAN

Inoculum		Ratio of virus carriers	Last day after feeding of virus isolation from stool	Ratio of positive antibody reactors
Type	Range of PD_{50} or TD_{50}			
2	3 1–5 8	47/87*	23	86/87†
1	3 2–5 5	15/15*	70	15/15

* Complete absence of clinical signs attributable to ingestion of virus
† Eight individuals in one trial developed homologous antibodies upon second feeding of virus

TABLE 2
RELATION BETWEEN INFECTIOUS DOSE FED AND INCIDENCE OF INTESTINAL CARRIAGE IN INDIVIDUALS WHO RECEIVED THE TN STRAIN OF POLIOMYELITIS VIRUS

Pool No	Mouse* PD_{50} of dose fed	Number of Individuals	Ratio of intestinal virus carriers	No of individuals from whose feces virus was isolated and No of isolations				
				1	2	3	4	5
16	5 8	6	6/6		2	3		1
		1†	1/1	1				
	5 3	1	1/1				1	
	4 8	1	0/1					
19	5 3	1	0/1					
24	5 3	1	1/1		1			
	5 0	8	4/8	2	1		1	
21	3 6	2	0/2					
23	3 1	5	3/5	2	1			
		1†	1/1	1				
31	4 4	53	26/53	14	5	4	2	1

* I C
† Type 2 antibodies present before feeding of virus

The association between the infectious dose of type 2 virus and the frequency of its intestinal isolation was also studied An over-all analysis of the data presented in TABLE 2 indicates that the isolation of virus on one occasion only from a given subject was the most common situation Although it may be claimed that larger concentrations of virus in the inoculum—as, for instance, log of 5 8 PD_{50} in the first trial—gave rise to a more prolonged carrier stage, and a smaller dose—log 3 6 PD_{50}—seemingly failed to induce excretion of the agent, the number of subjects was too small and the variations in the above pattern were too frequent to permit definite conclusions It is certain, however, that type 2 virus is not one to lurk a long time in the human alimentary tract The contrast with type 1 is only too obvious, when one examines the data presented in TABLE 3 Here, it can be clearly seen that the virus, after lodging itself somewhere in the alimentary mucosa, underwent cyclical multiplication without any apparent interruption as judged by the constant presence of the agent in stools The highest concentration of fecal virus was observed during the first 14 days after feeding Slightly lesser amounts were isolated subsequently and, only 35 to 40 days after the virus had been ingested, stool

TABLE 3
CONCENTRATION OF TYPE 1 VIRUS (SM STRAIN) IN FECES OF TWO INDIVIDUALS

Individual No	Stools pooled days after ingestion of virus	TD_{50}* of stool suspension
1	1, 3	2 2
	4, 6, 7	2 2
	9, 11	3 2
	13, 14	2 7
	15, 17	2 5
	23, 24	1 6
	27	1 6
	34, 35, 37	<1 0†
	43	<1 0†
	45, 47, 49	<1 0†
	51, 52, 53	<1 0†
	55, 57, 59	1 0 or more
	62, 63, 64, 66	<1 0 Negative‡
	67, 69, 70	<1 0 Negative‡
	72, 74	<1 0 Negative‡
2	1, 3	3 9
	4, 5, 6, 7	2 9
	8, 9, 11	4 0
	12, 13, 14	3 2
	15, 16, 17	2 5
	25, 26, 27, 28	<1 0†
	30, 33, 34, 35, 37	<1 0†
	42, 43, 44	<1 0†
	45, 46, 47, 48, 49	<1 0†
	51, 52, 54	1 0 or more
	56, 57, 58, 59	1 0 or more
	62, 63, 64, 65	<1 0 Negative‡
	66, 67, 68, 69, 70	1 0 or more
	72, 73, 74	<1 0 Negative‡
	76, 77, 78, 80, 82	<1 0 Negative‡

* Fifty per cent end point in tissue culture (monkey kidney)
† Positive on blind passage
‡ Negative on blind passage

suspensions failed, on direct inoculation, to cause cytopathogenic effect in tissue cultures. Blind passages, however, resulted in the recovery of virus, which finally seemed to disappear on the 62-to-66th day of the trial in one case. The other individual was found still to excrete the virus on the 70th day after its administration.

It is difficult to predict whether the differences in the behavior of type 1 and type 2 viruses represented inherent characteristics of the two agents. Perhaps the fact that the SM strain, even in low concentration, was cytopathogenic for monkey kidney and, *eo ipso*, for human tissue,[3] whereas the TN virus was devoid of this property altogether, may have some bearing upon their fate in the human host. This problem will be discussed later.

The presence of virus in the blood following ingestion has been studied exhaustively in the case of the type 2 strain.[3] Sixty-one individuals were employed as test subjects and their sera examined for the first 12 days after the administration of virus. As seen from TABLE 4, although 30 subsequently became intestinal carriers and all reacted with antibody formation, no virus

TABLE 4

ABSENCE OF VIREMIA AFTER ORAL ADMINISTRATION OF POLIOMYELITIS VIRUS

Type of virus administered	Schedule of blood collections*	Ratio of intestinal carriers	Number of positive blood isolations
2	1-12	30/61	0/61
1	1-8	3/3	0/3

* Days after administration of virus

was isolated from their blood Blood samples were also drawn from three individuals who became carriers after the ingestion of type 1 virus[4] None had viremia.

[illegible]

after, TN virus was fed to two of them, and mixtures of type 1 and type 2 to the other six The administration of immune serum globulin did not prevent the development of active immunity against type 1 virus, and seemingly failed to interfere with the infection of the alimentary mucosa, as evidenced by the ratio of individuals who became carriers of the virus. The results were less [illegible]

may have enhanced the active response to the virus, as indicated by the unusually high rise in antibody titer

The pathogenicity of the virus for monkeys after its passage through the human alimentary tract was studied in several instances. TABLE 5 summarizes the data obtained in one experiment The material that provided the inoculum fed to two human subjects was titrated intracerebrally and intraspinally in [illegible] No paralysis was evidenced by animals that received [illegible] ntraspinally, 3 of the 10 [illegible] paralysis Passage of the virus through the intestinal tracts of two human subjects failed to increase the virulence of the virus All monkeys into which injections were made

TABLE 5

PATHOGENICITY FOR CYNOMOLGUS MONKEYS OF TYPE 1 VIRUS AFTER PASSAGE THROUGH HUMAN INTESTINAL TRACT

Material	TD_{50}/ml or gm	Route	Paralytic ratio of monkeys at dilutions of virus				
			10^{-0}	10^{-1}	10^{-2}	10^{-3}	10^{-4}
Inoculum	7 0	I C	0/4	0/4	0/4	0/4	0/4
		I S	2/5	1/5	0/3	0/4	
Human stool (A)	5 5	I C		0/4	0/4	0/4	
		I S		0/4	0/4	0/4	0/4
(B)	6 0	I S		0/4	0/4	0/4	

A and B signify two different subjects, who received the same inoculum

Table 6
Persistence of Homologous Antibodies after Oral Administration of Type 2 Virus

Individual No.*	Minimum neutralizing titer of serum† obtained months after oral administration of virus							
	2	3	9	12	41	47	48	50
2			2 05				2 05	
3	2 10					2 95		
9		2 95			1 75			
16				2 95				2 65
17			2 95				>3 40	

* The past history of each individual was described in a previous publication [1]
† pH color change test titers of antibody expressed in negative logs to the base of 10

directly into the spinal cord with the two stool suspensions remained symptom-free during a 30-day observation period These results were obtained with an inoculum containing one log less virus concentration (as measured by the TD_{50} per gm of stool) than in the original material used for feeding purposes It is doubtful if this lack of virulence could be attributed solely to the lesser amount of virus Further experiments, however, have to be made in a larger number of experimental animals

The summary of the clinical trials could not be closed without the mention of the most important aspect of the study: the persistence of immunity It has been noted previously[4] that type 2 antibodies persisted at the same level in the blood of 14 children for three years after original ingestion of the virus Sera of five of the subjects have now been examined four years after virus administration (TABLE 6) In the case of only one individual, No 9, was a decrease in antibody content noted, and that was slight In the other four, the antibody level remained the same (within the limits of experimental error)

Interference Between Type 1 and Type 2 Viruses in the Human Alimentary Tract

Reciprocal interference between types of poliomyelitis virus has been observed in tissue culture [6, 7, 8] It was not expected that interference would take place in such spacious grounds for virus multiplication as the human alimentary tract As seen in TABLE 7 the first individual who received equal amounts, in terms of infectious doses, of the SM (type 1) and the TN (type 2) strains orally, was found to excrete type 1 virus only, and developed antibodies solely against type 1 In the next series, two individuals were fed simultaneously the two viruses in the ratio of 9 1 in favor of type 2 Both subjects became carriers of type 1 virus, and both developed antibodies only against type 1 The dose of type 2 virus was subsequently increased to an amount 20-fold greater than that of type 1 The results, however were no more encouraging than in the two previous cases When the ratio of type 2 to type 1 virus in the inoculum was changed to 100 1, interference by type 1 virus was surmounted The subject in this case became a dual virus carrier and developed antibodies against both viruses In the next trial six individuals ingested a mixture of the two virus types, with type 2 present in an overwhelmingly larger amount Four of the six subjects developed antibodies against both types, two reacted only against

TABLE 7

INTERFERENCE BETWEEN TYPE 1 AND TYPE 2 VIRUSES IN HUMAN INTESTINAL TRACT

Ratio between strains fed type 1 type 2	No of individuals fed	Intestinal carriage		Antibody response	
		No of individuals	Type of virus excreted	No of individuals	Type of virus
1:1 (4 7)*	1	1	1	1	1
1:9 (4 8)	2	2	1	2	1
1:20 (4 7)	1	1	1	1	1
1:100 (3 7)	1	1	1	1	1
	1	1	2	1	2
1·1000 (3 2)		6	1	6	1
	6			4	2
— (4 1)	1	0	—	1	2

* Virus administered, expressed as logs of TD_{50} or PD_{50}

the type 1 strain. The TN subline fed to these individuals, however, differed from that used in the original feeding experiment (see below). It should also be pointed out that these six subjects, in contrast to the others who received dual feedings, were given an injection of gamma globulin simultaneously with

bodies against the homologous virus, although type 2 virus was not recovered from the feces of subject L Detection of this particular strain of type 2 virus in the excreta, however, may be a difficult task The same strain (and type 1 virus) was fed to subjects F, G, H, I, J, and K, four of whom developed anti-

type 2 and type 1, and developed antibodies against both

In individuals A, B, C, D, J, and K, interference between type 1 and type 2 took place None became carriers of type 2, and they developed antibodies only against type 1 It is interesting to note that, contrary to the observations of others,[9] not even transitory type 2 neutralizing antibodies were observed in these six individuals who developed antibodies against type 1.

In an attempt to explain the interference phenomenon, one has to enter the realm of speculation *A possibility that cannot be overlooked* is that the actual locus of multiplication of the virus is restricted to a very limited number of cells of one particular part of the alimentary mucosa Once occupied by the faster multiplying virus (type 1), these cells, numerically *small, do not support the* growth of type 2 virus It is difficult to believe that interference would take place if the entire population of cells of the alimentary mucosa were uniformly susceptible to the growth of poliomyelitis virus Judging by the length of the intestinal tract, there should be enough cellular elements to support the growth

TABLE 8

INTERFERENCE BETWEEN TYPE 1 AND TYPE 2 VIRUS IN HUMAN INTESTINAL TRACT

Ratio between strains fed Type 1 Type 2	Individual	Excretion of virus			Antibody response				
		Stools collected (days after feeding)	Type excreted	Days after feeding	Type of virus	Titers* days after viral feeding			
						7	14	21	30
1:1 (4.7)†	A	2, 3, 4, 8, 9, (11 + 12), 17, 19, 24, 30, 44	1	2–30	1	1.5	NT	2.1	1.9
					2	0	NT	0	0
1:9 (4.8)	B	(3, 5, 6), 8, 14, 21, 51	1	3–21	1	0.9	1.6	2.1	2.5
					2	0	0	0	0
	C	3, 6, 7, 12, 19	1	3–19	1	0	>1.2	1.6	1.5
					2	0	0	0	0
1:20 (4.7)	D	3, 4, 9, 10	1	3–10	1	0.3	NT	1.5	1.6
					2	0	0	0	Trace
1:100 (3.7)	E	3, 11, 17, 37	1	3, 11	1	0.9	1.3	1.1	2.5
			2	3, 11, 17	2	0	0.9	1.8	1.8
1:1000 (3.2)	F	1–74	1	1–59	1	1.0	1.0	1.3	1.3
					2	1.0	1.6	1.6	1.6
	G	1–74	1	1–58	1	0.7	1.3	1.6	2.2
					2	0.7	>1.6	2.5	2.5
	H	1–53	1	1–51	1	0.7	1.0	1.3	1.6
					2	1.0	1.3	1.6	1.6
	I	1–82	1	1–71	1	1.3	1.3	1.3	1.6
					2	0.9	0.9	0.9	1.3
	J	1–82	1	1–70	1	1.0	1.3	1.3	1.6
					2	0	0	0	0
	K	1–73	1	1–49	1	1.0	1.6	1.6	1.9
					2	0	0	0	0
— (4.1)	L	3, 5, (8, 9), 14, 21	None		2	0.9	NT	1.5	1.5
(4.0) —	M	4, 9, 11, 13, (16, 17), 20, 21, 30, (38, 40)	1	4–34	1	NT	1.3	2.1	2.1
(5.5) —	N	5, 7, 13, 28, 46, 70	1	5–46	1	0	0.3	1.5	1.5

* Expressed as log to the base of 10
† Amount fed expressed as log of TD_{50} or PD_{50}
( ) Signifies pooled stool specimens
NT Not tested

of more than one type of virus, unless there is a tremendous difference in the rate of multiplication of the two virus strains. This is hardly the case, as judged by the same time of appearance of antibodies against either of the strains after alimentary infection (see TABLE 8). Emphasis should also be placed on the difference in cytopathogenic properties for monkey renal epithelium of the two types of virus. The markedly cytopathogenic type 1 always gains the *upper hand over the noncytopathogenic type 2*.

It should be repeated that all these potential explanations are wholly speculative at present. A study is now under way of strains of virus isolated from the feces of individuals who ingested both types. These strains, which have been classified serologically as type 1, seem to possess otherwise rather unusual properties, and an attempt will be made to separate them into the purest pos-

TABLE 7

INTERFERENCE BETWEEN TYPE 1 AND TYPE 2 VIRUSES IN HUMAN INTESTINAL TRACT

Ratio between strains fed type 1 type 2	No of individuals fed	Intestinal carriage		Antibody response	
		No of individuals	Type of virus excreted	No of individuals	Type of virus
1:1 (4 7)*	1	1	1	1	1
1:9 (4 8)	2	2	1	2	1
1.20 (4 7)	1	1	1	1	1
1.100 (3 7)	1	1	1	1	1
	1	1	2	1	2
1.1000 (3 2)		6	1	6	1
	6			4	2
— (4 1)	1	0	—	1	2

* Virus administered, expressed as logs of TD_{50} or PD_{50}

the type 1 strain The TN subline fed to these individuals, however, differed from that used in the original feeding experiment (see below) It should also be pointed out that these six subjects, in contrast to the others who received dual feedings, were given an injection of gamma globulin simultaneously with the administration of virus This apparently had no untoward effect upon the development of immunity to type 1 virus Conversely, however, this procedure may have had some inhibiting action against the development of immunity to type 2 This purely speculative hypothesis will be investigated

More detailed data on the same subjects are presented in TABLE 8 Three control subjects (L, M, and N) received one type only, and all developed antibodies against the homologous virus, although type 2 virus was not recovered from the feces of subject L Detection of this particular strain of type 2 virus in the excreta, however, may be a difficult task. The same strain (and type 1 virus) was fed to subjects F, G, H, I, J, and K, four of whom developed antibodies against type 2 (and type 1), yet no type 2 virus was recovered from stools collected and processed daily for 70 consecutive days. In contrast, subject E, who ingested another subline of the TN strain, became an intestinal carrier of type 2 and type 1, and developed antibodies against both

In individuals A, B, C, D, J, and K, interference between type 1 and type 2 took place None became carriers of type 2, and they developed antibodies only against type 1 It is interesting to note that, contrary to the observations of others,[9] not even transitory type 2 neutralizing antibodies were observed in these six individuals who developed antibodies against type 1

In an attempt to explain the interference phenomenon, one has to enter the realm of speculation A possibility that cannot be overlooked is that the actual ... of cells

multiplying virus (type 1), these cells, ... growth of type 2 virus It is difficult to believe that interference would take place if the entire population of cells of the alimentary mucosa were uniformly susceptible to the growth of poliomyelitis virus. Judging by the length of the intestinal tract, there should be enough cellular elements to support the growth

TABLE 8

INTERFERENCE BETWEEN TYPE 1 AND TYPE 2 VIRUS IN HUMAN INTESTINAL TRACT

Ratio between strains fed Type 1 Type 2	Individual	Excretion of virus			Antibody response				
		Stools collected (days after feeding)	Type excreted	Days after feeding	Type of virus	Titers* days after viral feeding			
						7	15	21	30
1:1 (4.7)†	A	2, 3, 4, 8, 9, (11 + 12), 17, 19, 24, 30, 44	1	2–30	1	1.5	NT	2.1	1.9
					2	0	NT	0	0
1:9 (4.8)	B	(3, 5, 6), 8, 14, 21, 51	1	3–21	1	0.9	1.6	2.1	2.5
					2	0	0	0	0
	C	3, 6, 7, 12, 19	1	3–19	1	0	>1.2	1.6	1.5
					2	0	0	0	0
1:20 (4.7)	D	3, 4, 9, 10	1	3–10	1	0.3	NT	1.5	1.6
					2	0	0	0	Trace
1:100 (3.7)	E	3, 11, 17, 37	1	3, 11	1	0.9	1.3	1.1	2.5
			2	3, 11, 17	2	0	0.9	1.8	1.8
1:1000 (3.2)	F	1–74	1	1–59	1	1.0	1.0	1.3	1.3
					2	1.0	1.6	1.6	1.6
	G	1–74	1	1–58	1	0.7	1.3	1.6	2.2
					2	0.7	>1.6	2.5	2.5
	H	1–53	1	1–51	1	0.7	1.0	1.3	1.6
					2	1.0	1.3	1.6	1.6
	I	1–82	1	1–71	1	1.3	1.3	1.3	1.6
					2	0.9	0.9	0.9	1.3
	J	1–82	1	1–70	1	1.0	1.3	1.3	1.6
					2	0	0	0	0
	K	1–71	1	1–49	1	1.0	1.6	1.6	1.9
					2	0	0	0	0
— (4.1)	L	3, 5, (8, 9), 14, 21	None		2	0.9	NT	1.5	1.5
(4.0) —	M	4, 9, 11, 13, (16, 17), 20, 21, 39, (38, 40)	1	4–34	1	NT	1.3	2.1	2.1
(5.5) —	N	5, 7, 13, 28, 46, 70	1	5–46	1	0	0.3	1.5	1.5

* Expressed as log to the base of 10.
† Amount fed expressed as log of TD_{50} or PD_{50}.
‡ Signifies pooled stool specimens.
NT Not tested.

of more than one type of virus, unless there is a tremendous difference in the rate of multiplication of the two virus strains. This is hardly the case, as judged by the same time of appearance of antibodies against either of the strains after alimentary infection (see TABLE 8). Emphasis should also be placed on the difference in cytopathogenic properties for monkey renal epithelium of the two types of virus. The markedly cytopathogenic type 1 always gains the upper hand over the noncytopathogenic type 2.

It should be repeated that all these potential explanations are wholly speculative at present. A study is now under way of strains of virus isolated from the feces of individuals who ingested both types. These strains, which have been classified serologically as type 1, seem to possess otherwise rather unusual properties, and an attempt will be made to separate them into the purest pos-

sible clones, which will then be individually investigated. Delayed administration of type 1 virus in relation to type 2 will also be studied in another trial. This procedure, theoretically at least, should make it possible to overcome the interference phenomenon at will, unless it should cause type 2 virus to interfere with type 1. The presence of viruses in the mucosa of the pharynx will also be examined in order to determine whether this region is a possible locus of viral multiplication.[10] Finally, it would be of interest to find out if interference can be reproduced in the human alimentary tract between two strains of virus which are both either noncytopathogenic or cytopathogenic for human tissue grown in culture. *Anyone exploring questions like these should be allowed loopholes* and exceptions because of the obvious difficulty of the problems involved.

Criteria of Attenuation

The author comes now to the most hypothetical and least dogmatic part of his remarks: the discussion of criteria of attenuation, or, in other words, what makes a virus acceptable as a living vaccine? *The next two tables summarize* his views.

TABLE 9 deals with properties of the virus relative to the host species concerned. Characteristics of the virus have purposely been chosen that can be evaluated in the average poliomyelitis research laboratory, and consideration is restricted to strains administered by the oral route. This has meant the

subjective rather than a guarantee of objective verisimilitude. For instance, it is the author's belief that the pathogenicity of a given strain for rodents is immaterial as a criterion of attenuation. It would be preferable to use a strain which has been propagated in mice and not in cotton rats since, as was mentioned yesterday, particles virulent for monkeys seem to thrive particularly well in cotton rat CNS.

In the author's opinion, a virus, to be considered acceptable for human vac-

TABLE 9

ACCEPTABILITY OF ATTENUATED POLIOMYELITIS VIRUS TO BE USED AS LIVING VIRUS VACCINE—RATING ACCORDING TO PROPERTIES OF THE VIRUS

(Personal Credo of the Author)

Animal host, tissue culture, etc., or characteristics	Properties of the virus that make it	
	Acceptable	Nonacceptable
Rodent CNS	Pathogenicity immaterial	
Cynomolgus CNS		
Brain	Nonpathogenic	Pathogenic
Cord	Nonpathogenic	Pathogenic
Tissue culture		
Monkey epithelial or fibroblast	Preferably noncytopathogenic	Cytopathogenic
Human epithelial	Preferably noncytopathogenic	Cytopathogenic
Genetic stability of the virus	Pure* clone desirable	Mixed population

* No change in the population after passage through human intestinal tract.

cination, should be completely nonpathogenic for cynomolgus monkeys when injected intramuscularly, or intracerebrally or when ingested. It is difficult to measure the pathogenicity of a virus inoculated directly into the spinal cord, since nonspecific factors may give rise to signs not pathognomonic for poliomyelitis infection

The demand that the vaccine virus be devoid of cytopathogenic properties

as far as alimentary infection goes, it has been the authors' experience that strains noncytopathogenic for either monkey or human tissue and avirulent for monkeys are less likely to be found in human stools, and probably can never become contagious after human passage

The characteristic of homogeneity of the viral population is perhaps the most important one Dulbecco's method[11] enables one now to determine, in a more or less precise way, the purity of a strain It is possible to adopt, in continuation of the present daydreaming, the criteria of purity developed by Burnet[12] for the influenza viruses In poliomyelitis, this would involve titration of the virus on plates, and isolation of plaques from the highest (limited) dilution Two successive L.D titrations should result in virus populations showing fairly uniform characters Strains noncytopathogenic for monkey kidney may become pathogenic for tissues of other origins, or will have to be tested for homogeneity in a much less exact way in animals

Again, it should be emphasized that the adoption for human vaccination of a strain with one property or another has be be dictated more by the conscience of the individual investigator and his "potential" experience than by his actual experience

The acceptability of attenuated strains, according to the reaction of the human host, is summarized in TABLE 10 Under no condition can a virus causing either clinical signs or viremia be used for vaccination purposes It is relatively easy to agree on this point, since the incidence of viremia is apparently

TABLE 10

ACCEPTABILITY OF ATTENUATED POLIOMYELITIS VIRUS TO BE USED AS LIVING VIRUS VACCINE RATING ACCORDING TO REACTION OF THE HUMAN HOST

(Personal Credo of the Author)

Sign or symptom	Acceptance as a vaccine		
	Ideal	Passable	Nonacceptable
Clinical signs	Absent	Absent	Present
Viremia	Absent	Absent	Present
Fecal excretion of virus	Absent	Occasional*	Frequent
Antibody response	Good	Good	Occasional

* Depending on whether or not excreted virus is contagious

high in naturally occurring infections in individuals who show *no an*
For instance, failure to recover TN virus from the blood *streams of*
cipients mentioned before (TABLE 4) indicates the general inability of
ticular strain to cause viremia and, *eo ipso*, paralysis—if you belie
recently proposed concept of pathogenesis[11] An ideal vaccine virus s
be found in fecal excretion. But it may be difficult to find three stra
senting all types which will cause infection of the alimentary mucosa,
by antibody response, but which, at the same time, either become c
before excretion or cannot be demonstrated in the excreta If, then,
is excreted by the human subject, it should remain noncontagious, e
cause of its inherent properties or because of very low concentration in
material Otherwise, the excreted virus would have to be studied an
pathogenic properties, and these investigations repeated after 'nat
artificial transfers through one, two, or three human passages Even
beloved argument of the opponents of live virus vaccination in genera
repeated, "*How do we know that the virus will not 'revert' to its virul*
after 10 or 20 serial passages in the human host?" It would be rather
ing if one could say in reply that, like the 17D strain of yellow fever, t
ticular strain of poliomyelitis was found to be noncontagious within th
of the investigation.

The importance of antibody response is obvious to everybody, and i
bly the only characteristic beside lack of clinical signs acceptable to one

These criteria for attenuation are not presented in a dogmatic fashi
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Now, a few words about future steps in live virus immunization

lation groups, then—giving a hint to Doctor Wilson—*by 1984 the inm*
asylums and of prisons in the United States will become the only two gr
society *permanently* immunized against poliomyelitis Paradoxically sp
a man who may have had the misfortune to be sentenced for life will be
the great chance of being protected forever against poliomyelitis.

In the author's opinion, it is time now to attempt "breaking through t
barrier" and to apply live virus immunization to the normal populatio
such trials, one should resist the sheer pressure of numbers, which goes
with a deplorable shrinkage of qualified observations, and resort to a
If the trial is restricte
on will be easie

Strains fulfilling some of the above criteria of attenuation are now ava
for such a trial, but their choice should be made neither by an individual n
any one organization but by a committee acting both in the interests c
advancement of science and the protection of the public.

If certain scientists are still haunted by the specter of live virus vaccines, they had better adopt, toward this new era of immunization, an attitude similar to that of Horatio announcing the appearance of Hamlet's father's ghost: "Look, my lord! it comes."

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high in naturally occurring infections in individuals who show no *antibodies*[11] For instance, failure to recover TN virus from the blood streams of the 61 recipients mentioned before (TABLE 4) indicates the general inability of this particular strain to cause viremia and, *eo ipso*, paralysis—if you believe in the recently proposed concept of pathogenesis[14] An ideal vaccine virus should not be found in fecal excretion But it may be difficult to find three strains representing all types which will cause infection of the alimentary mucosa, followed by antibody response, but which, at the same time, either become destroyed before excretion or cannot be demonstrated in the excreta. If, then, the virus is excreted by the human subject, it should remain noncontagious, either because of its inherent properties or because of very low concentration in the fecal material. Otherwise, the excreted virus would have to be studied anew for its pathogenic properties, and these investigations repeated after "natural" or

after 10 or 20 serial passages in the human host?" It would be rather comforting if one could say in reply that, like the 17D strain of yellow fever, this particular strain of poliomyelitis was found to be noncontagious within the limits of the investigation

The importance of antibody response is obvious to everybody, and is probably the only characteristic beside lack of clinical signs acceptable to one and all

These criteria for attenuation are not presented in a dogmatic fashion, but only as a basis for discussion One should always be reminded of a statement of Bertrand Russell that "man is a credulous animal, and must believe something, in the absence of good grounds for belief, he will be satisfied with bad ones"

Most of

Other

If the

research work continues at the present pace and remains limited to these population groups, then—giving a hint to Doctor Wilson—by 1984 the inmates of asylums and of prisons in the United States will become the only two groups of

In the author's opinion, it is time now to attempt breaking through [illegible] barrier" and to apply live virus immunization to the normal population In such trials, one should resist the sheer pressure of numbers, which goes along with a deplorable shrinkage of qualified observations, and resort to a limited

for such a trial, but their choice should be made neither by an [illegible] any one organization but by a committee acting both in the interests of the advancement of science and the protection of the public.

If certain scientists are still haunted by the specter of live virus vaccines, they had better adopt, toward this new era of immunization, an attitude similar to that of Horatio announcing the appearance of Hamlet's father's ghost: "Look, my lord! it comes."

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IMMUNIZATION OF CHIMPANZEES AND HUMAN BEINGS WITH AVIRULENT STRAINS OF POLIOMYELITIS VIRUS*

By Albert B Sabin

Children's Hospital Research Foundation, University of Cincinnati College of Medicine, Cincinnati, Ohio

Infective poliomyelitis viruses that are to be considered as candidates for human immunization must be avirulent in the sense that they are harmless for human lower motor neurons and, of course, also in other respects Since chimpanzees are most closely related to human beings, there is reason to believe that viruses that are harmless after inoculation into the gray matter of the spinal cord of chimpanzees may also be harmless for human lower motor neurons In the preceding communication,[1] I described the properties of strains of the three types of poliomyelitis virus that are avirulent by spinal inoculation in chimpanzees. Now I should like to present data on the behavior of such strains after oral and intramuscular administration in chimpanzees, as well as on some preliminary tests in human volunteers

TABLE 1 presents data that indicate that poliomyelitis viruses that are modified by cultivation in nonnervous tissue, whether of human or cynomolgus origin, possess a greater capacity for oral infectivity in chimpanzees than in cynomolgus monkeys. Thus the feeding of 15 ml. of the Brunhilde strain propagated in human tissue by Enders, Weller, and Robbins produced antibody in only 2 of 20 cynomolgus monkeys, while all 4 chimpanzees which received only 5 ml of the same culture fluid developed antibody.[2] The Mahoney, KP33 variant propagated in cynomolgus kidney tissue and administered in a 32-times greater dose than the Brunhilde strain in terms of tissue culture infective doses produced antibody in 8 of 20 monkeys, while much smaller amounts *sufficed to produce an immunogenic infection in chimpanzees* The same difference in response of cynomolgus monkeys and chimpanzees is also apparent after intramuscular injection The data shown in TABLE 2 indicate that $10^{4.5}$ to $10^{1.7}$ TCD_{50} *of the Brunhilde and Mahoney strains of the type 1 virus*, contained in as little as 0 001 ml of culture fluid, sufficed to immunize all chimpanzees, but only a small proportion of cynomolgus monkeys A similar small amount of the *type 3, Leon virus, however*, was also without effect in chimpanzees

The data presented in TABLE 3 show the antibody response of chimpanzees to a single feeding of three strains of type 1 virus. With the exception of one sick chimpanzee that died of intercurrent disease in six weeks, four others developed ... of ... nse ... ilar ... the amounts of types 2 and 3 viruses The antibody titers were generally ... the same order of magnitude at 12 weeks as at 4 weeks. The 4 chimpanzees that developed antibody, all excreted virus—2 of them for as long as 84 days. When only *0.001 ml* of the same culture fluid was fed to three chimpanzees, only one developed antibody, but not until four weeks after feeding, although

* Aided by a grant from the National Foundation for Infantile Paralysis, Inc, New York N Y

TABLE 1

COMPARATIVE ORAL INFECTIVITY IN CYNOMOLGUS MONKEYS AND CHIMPANZEES OF TYPE 1 POLIOMYELITIS VIRUSES MODIFIED BY CULTIVATION IN NONNERVOUS TISSUE

Strain of virus	Inoculum		No. developed antibody	
	Ml fed	$Log_{10}TCD_{50}$	Cynomolgus	Chimpanzee
Brunhilde, Enders *et al* (Human tissue)	15	6.9	2/20	—
	5	6.4	—	4/4
Mahoney, KP 33 (Cynomolgus kidney)	7.5	8.4	8/20	—
	0.5	7.2	—	4/5
	0.001	4.5	—	1/3

TABLE 2

VARYING CAPACITY OF SMALL AMOUNTS OF POLIOMYELITIS VIRUSES TO PRODUCE IMMUNOGENIC INFECTION AFTER INTRAMUSCULAR INJECTION IN CYNOMOOGUS MONKEYS AND CHIMPANZEES

Viruses Segregated by Cultivation in Nonnervous Tissue

Type and strain of virus	Am t inoculated		No. developed antibody	
	Ml	TCD_{50} Log_{10}	Cynomolgi	Chimpanzees
1 *Brunhilde* (Enders *et al*) Human tissue	1.0	5.7	3/9	—
	0.1	4.7	3/10	4/4
1 *Mahoney*, KP 33, Cynomolgus kidney	0.01	5.5	5/5	—
	0.001	4.5	2/9	3/3
3-*Leon*, KP 34, Cynomolgus kidney	0.1	6.4	4/5	3/3
	0.01	5.4	2/5	—
	0.001	4.4	2/5	0/2
	0.00,001	2.4	1/5	0/3

virus excretion in amounts of about 10^4 TCD_{50} per gram of feces was already evident at seven days after feeding, and continued for at least six weeks. The results with a tissue culture derivative of the mouse-adapted Mahoney virus of Li and Schaeffer (LSa) indicate that some strains may be very poorly immunogenic in chimpanzees, even though they can multiply in the alimentary tract. Thus two of the three chimpanzees that received 10^7 TCD_{50} already excreted virus in amounts of $10^{2.5}$ to 10^3 TCD_{50} per gram of feces at seven days, but one of them still had no demonstrable antibody in six weeks. The third chimpanzee, in whose feces no virus was detected, developed a trace of antibody at one and two weeks but none was demonstrable at three, four, five, and six weeks. On the other hand, all four chimpanzees that received only $10^{6.4}$ TCD_{50} of the Brunhilde strain developed antibody, even though only one of them excreted virus, and that at a low level.

The test carried out with the type 2, YSK, KP51 virus shown in TABLE 4 may have been complicated by the fact that the first three chimpanzees had spontaneously acquired type 3 antibody, which may have been due to recent infection. The animals were used shortly after arrival from Africa, and one of the chimpanzees from the same shipment had a spontaneous poliomyelitis infection on arrival that yielded a monkey-intracerebrally-virulent type 3 virus. Two

TABLE 3

ANTIBODY RESPONSE OF CHIMPANZEES TO FEEDING OF MONKEY INTRACEREBRALLY AVIRULENT TYPE 1 VIRUSES

Virus fed	Dose	Chimpanzee No	Antibody titer—weeks after single dose: Pre	1	2	3	4	6	12
Mahoney, KP 33, Avirulent for chimpanzees by spinal route	0 5 ml $10^{7}\ ^{2}TCD_{50}$	1 (6876)	0	0	0	0	0	0 ← Died—intercurrent disease	
		2 (6877)	0	0	4	200	64	20, 13	130
		3 (6881)	0	0	20	64	64	32; 40	130
	As above + $10^{7}\ ^{2}TCD_{50}$—type 2 and $10^{7}\ ^{4}TCD_{50}$—type 3	4 (6872)	0	0	32	320	64	32, 64	64
		5 (6873)	0	0	±	32	320	64, 64	64
	0 001 ml $10^{4}\ ^{4}TCD_{50}$	6 (7343)	0	0	0	0	0	0	
		7 (7404)	0	0	0	0	0	0	
		8 (7406)	0	0	0	0	4	200	
Mahoney, "LS a". Segregated by terminal dilution in monkey kidney tissue culture	1 ml $10^{7}TCD_{50}$	9 (7336)	0	0	0	0	0	0	
		10 (7344)	0	0	0	0	0	32	
		11 (7409)	0	trace	±	0	0	0	
Brunhilde, "Enders et al" Human tissue culture	5 ml $10^{6}\ ^{4}TCD_{50}$	12 (4860)	0	0	0	3	20	50*	
		13 (4861)	0	0	0	32	200	50*	
		14 (4862)	0	0	0	3	32	50*	
		15 (4868)	0	0	0	0	4	100*	

* These are titers at five weeks rather than at six weeks

TABLE 4

ANTIBODY RESPONSE OF CHIMPANZEES TO FEEDING OF CHIMPANZEE-AVIRULENT TYPE 2 VIRUS (YSK, KP 51)

Dose	Chimpanzee No	Antibody titer—weeks after single dose: Pre	1	2	3	4	6	12	26
1 ml $10^{7}\ ^{2}TCD_{50}$	1* (6883)	0	0	0	0	0	0 Died—intercurrent disease		
	2* (6884)	0	0	0	0	3	±	6	
	3* (6882)	0	0	25	64	250	640, 640	130	
above + $10^{7}\ ^{2}TCD_{50}$—type 1	4 (6872)	0	0	200	64	200	200, 64	400	
and $10^{7}\ ^{4}TCD_{50}$—type 3	5 (6873)	0	0	100	2,000	320	320, 400	64	

* These chimpanzees had spontaneously acquired type 3 antibody which may have been due to recent infec-
Possibility of modification of type 2 infection must be considered

ier chimpanzees that had been in the laboratory for many months, however, d had no antibody for any of the three types, were used at the same time and veloped antibody in high titer, even though the same dose of type 2 virus was xed with equal amounts of types 1 and 3 virus Only one of the four chimnzees that developed antibody excreted virus, and that on only one occasion d in very small amount

The chimpanzees used for the test with the type 3 virus had no antibody for y of the three types to begin with The data shown in TABLE 5 indicate that

TABLE 5

ANTIBODY RESPONSE OF CHIMPANZEES TO FEEDING OF CHIMPANZEE-AVIRULENT TYPE 3 VIRUS (LEON, KP 34)

Dose	Chimpanzee No	Antibody titer—weeks after single dose									
		Pre	1	2	3	4	6	12	14	16	18
1 ml $10^{7.4}$ TCD_{50}	1 (6871)	0	0	4	32	320	320, 64	64			
	2 (6874)	0	0	64	320	250	320, 64	64			
	3 (6880)	0	0	3	6	32	64, 64	64			
As above + $10^{6.4}$ TCD_{50}—type 1 and $10^{7.3}$ TCD_{50}—type 2 + $10^{7.4}$ TCD_{50}—type 3 at 14 weeks	4 (6872)	0	0	0	0	0	0	0	0 ↑	0	64
	5 (6873)	0	0	0	0	0	0	0	0 ↑ virus	3	20
0.001 ml $10^{6.4}$ TCD_{50}	6 (7404)	0	0	0	0	0	0				
	7 (7405)	0	0	0	0	0	0				
	8 (7406)	0	0	0	0	0	0				

one dose of $10^{7.4}$ TCD_{50} produced antibody in the indicated titers in all three chimpanzees within two weeks, and all excreted virus for short periods of time. However, in two other chimpanzees that were used at the same time and re-

antibody development

The data shown in TABLE 6 indicate that the antibody response resulting from intramuscular infection with this strain of type 3 virus is not only of a lower order than that resulting from oral infection, but also does not persist Two of the 3 intramuscularly inoculated chimpanzees lost practically all of their

TABLE 6

DIFFERENCE IN ANTIBODY RESPONSE OF CHIMPANZEES TO FEEDING AND INTRAMUSCULAR INJECTION OF CHIMPANZEE AVIRULENT TYPE 3 VIRUS (LEON, KP 34)

Route and dose	Chimpanzee No	Antibody titer—weeks after virus							
		Pre	1	2	3	4	6	8	12
Oral 1 ml $10^{7.4}$ TCD_{50}	1 (6871)	0	0	4	32	320	320, 64		64
	2 (6874)	0	0	64	320	250	320, 64		64
	3 (6880)	0	0	3	6	32	64, 64		64, 64
	4* (6872)	0	0	0	32	64	200		
	5* (6873)	0	0	3	32	20	32		
Intramuscular, 0.1 ml $10^{6.4}$ TCD_{50}	6 (7335)	0	6	4	20	20	4	3	0†
	7 (7336)	0	10	10	10	4	3	0?	0?†
	8 (7337)	0	20	32	6	4	4	4	4†

* These chimpanzees had previously been fed a mixture of all three types of virus and failed to respond with type 1 antibody When they were again fed type 3 virus 14 weeks later they had high titers of types 1 and 2 antibody The other chimpanzees (Nos 1, 2, 3, 6, 7 and 8) had no antibody for any type of poliomyelitis virus at the time of virus administration

† These titers are for 11 weeks after inoculation of virus

TABLE 7

EXCRETION OF VIRUS AND VIREMIA AMONG CHIMPANZEES INFECTED BY ORAL OR INTRAMUSCULAR ADMINISTRATION OF CHIMPANZEE-AVIRULENT POLIOMYELITIS VIRUSES

Route	Type of virus	Number developed antibody	Number excreted virus	Number viremia 5, 7, 10, 14 days
Oral	1	9	6	0
	2	4	1	0
	3	5	4	0
	Total	18	11	0
Intramuscular	1	9	0	0
	2	2	0	0
	3	5	0	0
	Total	*16*	*0*	*0*

antibody by 11 weeks This was not found to be the case in chimpanzees inoculated intramuscularly with a similar dose of type 2 virus—in these, the antibody developed in high titer and persisted at a high level, as it did in those that received the virus by mouth These observations indicate that not all strains are equally suited for immunization by parenteral routes, and that comparative studies on oral versus parenteral modes of immunization must take into account not only the initial antibody response but also its duration.

A summary of the tests for viremia and virus excretion in the chimpanzees that developed antibody is shown in TABLE 7. The tests for viremia carried out at 5, 7, 10, and 14 days after administration of virus were negative in all chimpanzees Whereas virus excretion was detected in 11 of the 18 oral infections, none was demonstrable in the 16 intramuscular infections. Thus absence of readily demonstrable viremia is another important distinguishing feature between the avirulent and virulent poliomyelitis viruses[3] The fact that parenteral administration of the avirulent viruses results in what appears to be a closed infection, in the sense that no virus is excreted, may be one of the points in favor of using this route for immunization, provided strains can be found that will produce a durable immunity

The character of the virus excreted by the chimpanzees was tested by intracerebral inoculation in monkeys in two ways (1) by inoculation of a 10 per cent stool suspension containing from $10^{3.5}$ to $10^{5.5}$ TCD_{50} of virus, and (2) by inoculation of the virus growing out from the stools in tissue culture These tests revealed that after feeding of the intracerebrally avirulent, Mahoney KP33 strain, a small proportion of the progeny that developed in the alimentary tract of the chimpanzees were intracerebrally virulent for monkeys Even when virus excretion continued for 84 days, these particles did not become dominant, nor did a second passage of the original stools in the alimentary tract of chimpanzees establish them as the dominant population It is especially note-[illegible] to the virulence of the

[illegible] it in

[illegible] scu-

larly inoculated cynomolgus monkeys, nor did they produce [illegible] neu-

FIGURE 1. Antibody response to single feeding of chimpanzee-avirulent poliomyelitis virus in human volunteers.

ronal lesions after direct spinal inoculation in three chimpanzees. It is also noteworthy that such intracerebrally virulent mutants were not encountered in the chimpanzees fed the Brunhilde strain or the poorly immunogenic tissue culture derivative of the Li-Schaeffer mouse-adapted Mahoney virus.

The purpose of the first preliminary tests on human volunteers was to determine whether chimpanzee-avirulent virus would multiply in the human alimentary tract. The first tests were done on three individuals who had low titers of antibody, and the data are shown in FIGURE 1. The man who swallowed 1 ml. of type 2 culture fluid excreted virus at 7 and 10 days at levels of $10^{2.3}$ to $10^{4.5}$ TCD_{50} per gram of feces and his type 2 antibody increased from a titer of 1:32 to 1:3200 at which it has persisted for about six months. There was no significant change in the titers of type 1 and type 3 antibody. The excreted virus contained no intracerebrally virulent mutants. Two men swallowed 1 ml. of the type 3 virus. The man who had a type 3 antibody titer of 1:32 to begin with did not excrete virus and exhibited no change in titer over a period of six months. The other man whose type 3 antibody fluctuated from 1:3 to 1:15 during the preceding months, excreted virus at seven days at a level of $10^{2.2}$ TCD_{50} per gram of feces and showed an increase in the type 3 antibody to a titer of 1:320 which gradually returned to a level of 1:32 which is still present at six months. The excreted virus contained no intracerebrally virulent mutant.

Tests are now in progress on a larger number of human volunteers who have no demonstrable antibody for the type of administered virus.[4] The purpose of these and future tests is to obtain a quantitative estimate of the relative susceptibility of human beings and chimpanzees to immunogenic infections with small amounts of chimpanzee-avirulent viruses, as well as to test for viremia and the amount and character of excreted virus.

In conclusion, I should like to say that I consider it unwise to have preconceived notions about the duration of immunity produced by living avirulent or killed virus vaccines. It is obvious that both will have to be studied under carefully controlled conditions over a period of many years before we will know

TABLE 7

EXCRETION OF VIRUS AND VIREMIA AMONG CHIMPANZEES INFECTED BY ORAL OR INTRAMUSCULAR ADMINISTRATION OF CHIMPANZEE-AVIRULENT POLIOMYELITIS VIRUSES

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panzees establish them as the dominant population

worthy that this does not represent a reverse mutation *to the virulence of the* parent Mahoney virus, since the intracerebrally virulent particles grown out in tissue culture from chimpanzee stools did *not produce paralysis in* intramuscularly inoculated cynomolgus monkeys, nor did they produce paralysis or neu-

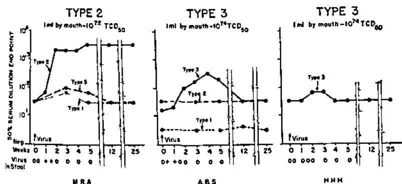


FIGURE 1 Antibody response to single feeding of chimpanzee avirulent poliomyelitis virus in human volunteers

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the answer Although we have learned a great deal in the last two years, I think that we are at the beginning rather than at the end of our studies of this important problem

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SUMMARY AND REVIEW OF POLIOMYELITIS IMMUNIZATION

By Thomas Francis, Jr.
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I wish I had the elegance of expression to discuss adequately the excellent foregoing studies on poliomyelitis immunization Although the basic contents are familiar, the details presented were entirely new, so that I was cold, too, on receiving them It is difficult to take a position of critic with respect to the material because each writer has so completely built his protective fence by labelling much of his discussion speculation But then I can feel free to indulge in speculation, too

Doctor Koprowski has fortunately provided a fine text with his references to Bertrand Russell and Aristotle. It happens that I recall, too, that in one of his *Unpopular Essays*, Russell pointed out that we frequently remember a man for the brilliant sayings ascribed to him and forget many of his absurdities Despite his other brilliance, Aristotle, according to Russell, belonged to the group that believed that women had fewer teeth than men, whereas all he needed to do was to look in Mrs Aristotle's mouth and make an observation This serves only to emphasize that, however deeply we may be impressed by our opinion, the evidence gained by putting the thesis to test is, in the end, the deciding factor

This may be, however, an appropriate time to consider the concepts involved in the two approaches to immunization against poliomyelitis that are represented in the data contained in the papers presented in this monograph We grow accustomed to muddied water, just as the cryptobranchs do, but one may try to look at the situation clearly in terms of the immunologic principles involved

One approach follo[illegible]

tiny First of all, the pathogenesis of the diseases must be considered In the case of yellow fever, there is no evidence that inactivated virus will not induce good immunity in man, and, because of the viremic character of the illness, I would wager that it could Furthermore, proper gamma globulin would probably protect Measles and hepatitis are outstanding invitations to vaccination with inactive virus by virtue of the extensive evidence that antibodies furnished by gamma globulin are highly protective The studies of Kempe with gamma globulin in the prevention of small pox again indicate that infection is not a requisite for protection Similarly, the ready demonstration of protection of experimental animals against poliomyelitis by gamma globulin, as well as the field studies of Hammon and his colleagues in man, again support the concept that antibodies alone can prevent this disease

The two outlooks are, then, simply this inactive virus vaccine is apparently a test of the straightforward hypothesis that antibody induced by the adminis-

tration of antigen can provide protection without subjecting the recipient to harmful effects of even the inapparent infection The other, through the use of modified active virus, seeks to induce antibody formation, but wishes to add some undesignated advantage derived from assumedly harmless infection (I am not certain that any significant infection may not create undesirable tissue reactions) *The solidity of immunity after natural infection is quite apparent* with certain virus diseases. With others, its impermanence is illustrated by recurrences without added infection. The advantages of infection have been variously ascribed to tissue immunity, persistence of immunogenic virus, and increased or widely dispersed dosage, and the latter seems perhaps the most important.

One is bound to be influenced by his background and experience. Influenced by one of our early studies that demonstrated the capacity of purified pneumonococcus polysaccharide to induce specific immunity in man, I favor an immunologic outlook which would avoid infection and seek the active immunizing principles. *This, I think, is the line of future immunologic advance rather* than to create an inapparent infection that appears harmless, perhaps only because we are working at such a gross level for detection of injury

I might point out that our first studies with influenza vaccine were made by injection of active virus, later with inhalation of active virus The evidence changed our approach because we obtained better results with injection of inactive virus than by intranasal administration of active virus.

Which of these approaches to poliomyelitis will be the more effective is, then,

Continuing studies on all these lines are highly desirable, and conclusions will be based on the evidence obtained, not upon the weight of opinion

It is ap-
alimentary
is not pre-
vented Diphtheria is a striking example of the fact, however, that prevention of active disease reduces the reservoir but does not necessarily eliminate the organism Doctor Sabin's data indicate that intramuscular inoculation of his modified agents requires just about as much active virus to induce antibodies *with regularity as is required* with inactive virus If there is no multiplication and the route is not the "presumed" natural one, one wonders what advantage this can have over well-prepared inactive material Doctor Koprowski, in

ements of active virus for immunization by the ali-
feces after feeding
oncealed or masked
in some way? There are those who suggest that modified virus given in this
be distributed and maintained by the usual

but that progress in the field is an unending pursuit

SUMMARY AND FORECAST: POLIOMYELITIS IN 1984

By G. S Wilson

Public Health Laboratory Service (England and Wales), Medical Research Council, London, England

I find myself in an honored but very precarious position When Doctor [illegible]

experience of the disease, and such indirect knowledge as I have gained from reading and from discussion with my colleagues is not such as to prejudice me consciously in favor of or against any particular view Unfortunately, ignorance is not the only qualification needed Had I the necessary imaginative insight, I should be able to tell you how poliomyelitis would behave during the next thirty years, whether left uncontrolled or attacked with all the vigor characteristic of your people But I do not have such insight, and even the [illegible]

so complex that he would be a bold man who would dare to predict the part played by poliomyelitis in the year 1984 All I can hope to do is to attempt to pick out what, to my mind, are the high lights in the picture, and to suggest which parts require more detailed brushwork Arnold Bennett used to say that no man could be a good critic who was not himself a creative artist, and I must therefore beg you to excuse me if my lack of expert knowledge of your subject leads me into those gaucheries which are characteristic of the inexperienced

Cultivation and Recognition of the Virus

I need say little about the methods of cultivating and recognizing the virus [illegible]

Cell Susceptibility

The papers in this monograph dealing with cell susceptibility have already been summarized by Doctor Luria Ample evidence is now at hand to demonstrate the predisposing effect of injections of certain prophylactic agents and of tonsillectomy but it is still doubtful how far prophylactic injections act by localizing an active infection, and how far by converting into clinical disease

an infection that would otherwise have remained latent. Do such injections, for example, play no more than the part of a fixation abscess in a staphylococal septicemia, or have they the same determinant effect that calcium ions have in activating washed tetanus spores in the guinea pig or that splenectomy has in activating *Hemobartonella* in the rat? Doctor Paffenbarger's results indicate that tonsillectomy falls into this latter category, but the mechanism by which it acts still awaits elucidation.

Inhibition of Growth of the Virus

I am a little surprised that, though much has been said about ways of altering the susceptibility of the cell, there has been little or no mention of ways of alter- [illegible] be [illegible] ne blue interfere with the growth of rickettsiae in egg culture and that, in man, chloramphenicol has an even stronger effect. There is evidence that members of the lymphogranuloma-psittacosis group exhibit some degree of susceptibility to the nitroacridines, the quinoxalines and, still more, the tetracyclines In the [illegible] lism of ribonucleic acid in the infected cell Moreover, the injection of sodium fluoroacetate into mice shortly before the intracerebral inoculation of Lansing virus is reported to inhibit the early stage of growth of the virus and to delay the onset of the disease Further, according to Shope, the antibiotic helenine inhibits the *in vivo* reproduction of the Columbia SK and the Semliki Forest viruses in mice Finally, we have the curious observation of Sanders, that the development of poliomyelitis may be prevented by the injection of detoxified cobra venom

These findings, though few and inconspicuous, are nevertheless sufficient to hold out hope that further investigations may not prove altogether fruitless I suspect that the real obstacle to success lies not so much in the impossibility of the end as in the means of reaching it During the past 20 years or so, thousands of chemical and mold products have been examined, resulting in the selection of not more than a score of substances having useful antibacterial activity No rapid screening method is yet available to carry out a similar quest on viruses Until a satisfactory method is devised, the discovery of antiviral substances applicable to human and veterinary medicine is likely to be slow.

Variations in Virulence

I have little to add to Doctor Hattie Alexander's summary on variations in virulence of the virus Studies of this type are of value partly because they improve our knowledge of the pathogenesis of the disease, and partly because they provide us with strains of a suitable degree of attenuation to be employed as living vaccines It seems to me unfortunate that so little attention is now

paid to the major problem of virulence and susceptibility. Modern bacteriologists spend much of their time in the infinitely tedious study of minor biochemi- [illegible]

has to be collected before this is possible Did not Henry James say that it takes a great deal of history to make a little literature? But my point is that we must continuously bear in mind the ultimate aim, that of explaining how and why infection occurs. Why is it that some organisms gain a lodgment in the tissues without giving rise to disease, and others, though pathogenic when artificially introduced into the tissues, are unable to spread from one host to another? These, and similar fundamental questions, are queries that I believe the virologists, with their growing mastery of technique, including their ability to study the intimate relation existing between the virus and the cell, may go a long way towards answering

Mode of Spread of Infection

The discussions in this monograph on the relation between the virus and the community have raised the problem of how poliomyelitis infection spreads This is indeed a puzzle Broadly speaking, there are two schools of thought The one, influenced by the apparent restriction of the virus to the immediate vicinity of the patient, insists that infection is limited to a small number of foci and spreads in a narrow stream The other, influenced by the widespread distribution of neutralizing antibodies in the population, believes that infection must also be widely disseminated The question really is. Does poliomyelitis behave like typhoid fever or cerebrospinal meningitis? The evidence at present is conflicting and difficult to interpret More information is required, particularly of a quantitative nature How often, for example, can case-to-case or case-to-carrier infection be established? The findings differ enormously Thus Casey, studying the outbreak in Alabama in 1941, traced a contact history between patient and patient in 80 per cent of the 121 cases observed On the other hand, in the 1950 outbreak at Dudley in Worcestershire, England, recorded by Lawson, case-to-case infection could not be demonstrated in a single one of the 63 cases that were noted Investigations might be directed profitably toward ascertaining the proportion of the population that develops neutralizing antibodies to the different antigenic types of virus in the absence of any known contact with patients suffering from the disease, in much the same way as Fox and his colleagues have done in Louisiana, and Hammon and his colleagues in the Philippines

We may say confidently that poliomyelitis does not resemble pulmonary tuberculosis, a disease in which all infection occurs from open cases and in which no infectious healthy carriers exist If complete segregation could be imposed on all patients suffering from the disease, tuberculosis could be stamped out But in poliomyelitis, our herd immunity, for the present at any rate, must rest on the same processes of latent infection and natural immunization as in diphtheria and scarlet fever Our task is to learn how to make use of this mechanism without paying too great a price in the form of clinical disease

Route of Access of Infection to the Body

This leads on to another question. Can we, in practice, control what Dudley called the velocity of infection? To answer this question we ... what form and by what route inf... body. Is it in the form of ... geal droplets inhaled by th... ...yelitis, in fact, an enteric or a respiratory infection? There is strong evidence pointing in both directions. In considering it, we must not lose sight of the ... the fact that ... before and ... to be infect... of s... ...ing this period of infectivity tha... ...s can be demonstrated in the pharyngeal secretions suggests that infection is essentially of the droplet type ...

... some weeks into convalescence. Does the patient's loss of infectivity depend simply on the disappearance of virus from the pharynx as soon as neutralizing antibodies are formed, or is there in addition, some change in virulence that lessens ... to host? Again, if the virus persists i... fully virulent and fully infective form, ... tious? Does this depend on the difference in the route of access to the body? We know that, in man, tubercle bacilli are far more likely to set up disease when inhaled than when ingested May the same thing hold true of poliomyelitis? The rhesus monkey is insusceptible to infection by the mouth, though it can be infected by the nose Cynomolgus monkeys and chimpanzees can be infected by the mouth, but even they are far more susceptible to infection by the intranasal route Possibly man is most likely to contract the disease when he inhales the pharyngeal secretions of the patient, and most likely to develop a latent immunizing infection when he ingests the excretal material of patients and healthy carriers

To resolve the anomaly, we need such an investigation as that ... Capps and ... ...al spread from the children without interfering with spread by the respiratory route I see no simple way of repeating this investigation on poliomyelitis, but methods must be devised for deciding what part each of these routes plays in the spread of infection

At the same time, some explanation should be sought for the peculiar seasonal incidence of the disease If infection occurs by the respiratory route, why do cases occur almost solely in the summer and autumn months? If it occurs by the enteric route, why should a disease that appears to be generally independent of carriage by water, milk, food, or flies occur in the summer, and not in the winter when human contact is most close?

Prophylactic Vaccination

Lastly, we must consider whether prophylactic inoculation can be used to raise the immunity of the [illegible]
in favor of vaccination [illegible]
disease, it provides us w[illegible]
evidence in favor of the occurrence of a viremic stage in the development of the disease adds enormously to our confidence in the correctness of this view. Admittedly, the controversy between the exponents of the viremic mode of pathogenesis and of the direct intraneural spread of the virus has still to be resolved, but what we have learned about the protective value of γ-globulin under experimental conditions, and of the association, under natural conditions, between the presence of type-specific neutralizing antibodies and resistance to the disease entitles us to aim at the production of a high level of humoral antibodies.

How this is to be attained, I do not profess to know, but we may draw upon our general [illegible]

tection it affords. Thus a living, slightly attenuated vaccine is more potent than a highly attenuated vaccine, and a vaccine prepared from a given strain is more potent when alive than dead. If, therefore, we are to establish a fairly [illegible]

and immunizing ability are not easy to find or to produce. In other diseases, only one or two such strains are available. Thus in anthrax, we have the Carbozoo strain, in tuberculosis, the Calmette-Guérin strain, in yellow fever, the 17D strain, in rinderpest, the Kabete strain, and, in rabies, the Flury strain. Even if we did find suitable strains for poliomyelitis, however, we [illegible]

pox, yellow fever, rinderpest, swine fever? The occurrence of multiple virus types adds greatly to the difficulty of successful vaccination. In the first place, there is no guarantee that, when protection has been afforded to the known types, a new type may not arise and cause unexpected havoc. Secondly, we are becoming aware only now, through the work of Glenny and Barr, of the difficulty of eliciting equally powerful responses to each of the multiple antigenic components of a vaccine. The reaction of the body to a mixed vaccine depends not only on the antigenic potency of each of the components, but on the pre-existing level of immunity towards them. If there is antibody to one of them, then the antibody response to the others may be partly or completely suppressed. Our experience of attempted protection by a formolized vaccine

against foot-and-mouth disease—a disease caused by at least three different types of virus—has not been fortunate, and we may find that the same thing holds true of poliomyelitis

If we decide to use a killed vaccine, we shall probably have to rely on the [illegible]

and that the disease may die out through lack of susceptible hosts in which to spread

On the whole, I think the outlook is hopeful. When we have succeeded in preparing a suitable vaccine and, at the same time, learned how to regulate the velocity of infection in relation to the degree of protection afforded by the vaccine, I believe that, for all practical purposes, we shall have solved the problem of poliomyelitis.

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